

- of 91

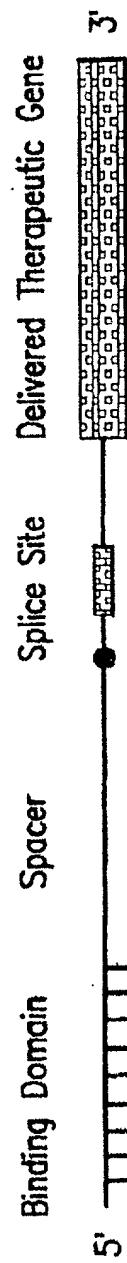
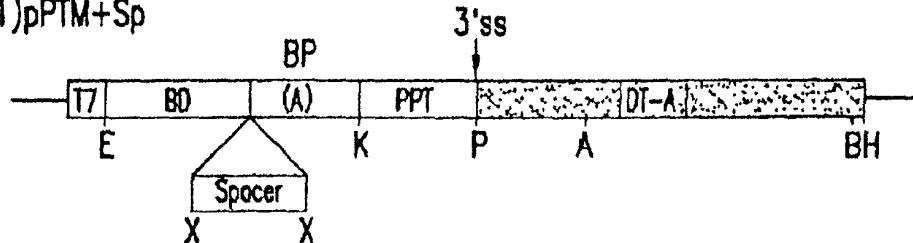


FIG. 1A

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(1)pPTM+Sp



(2)pPTM+Sp

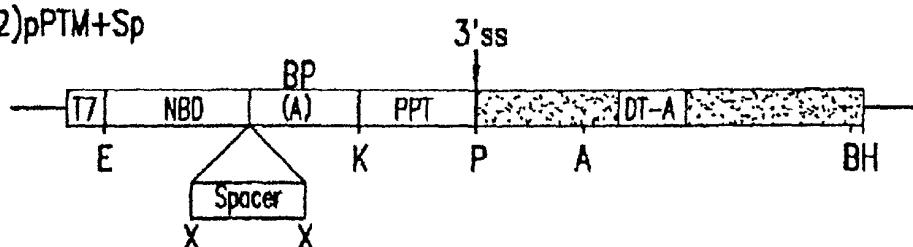


FIG.1B

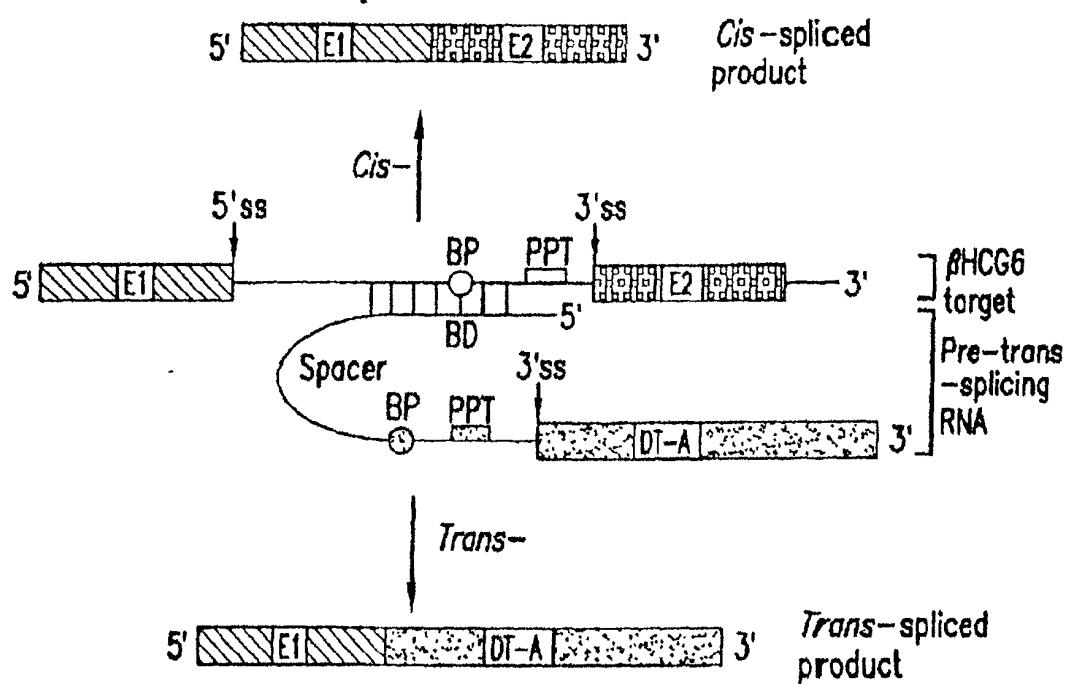


FIG.1C

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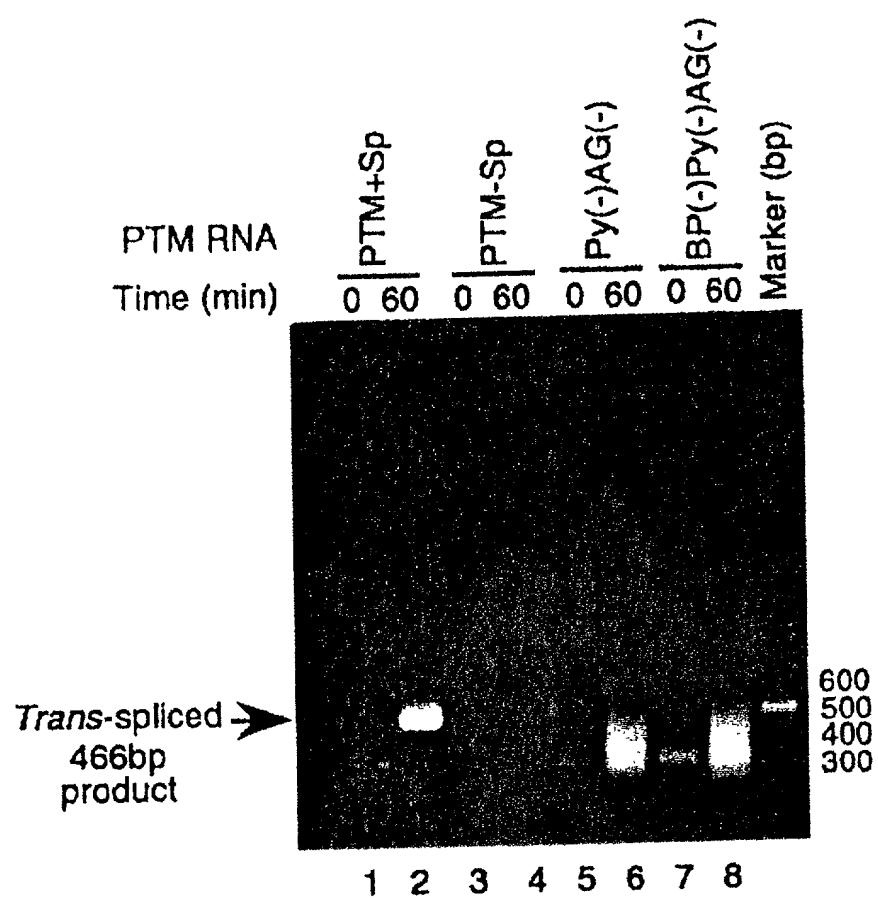


FIG.2A

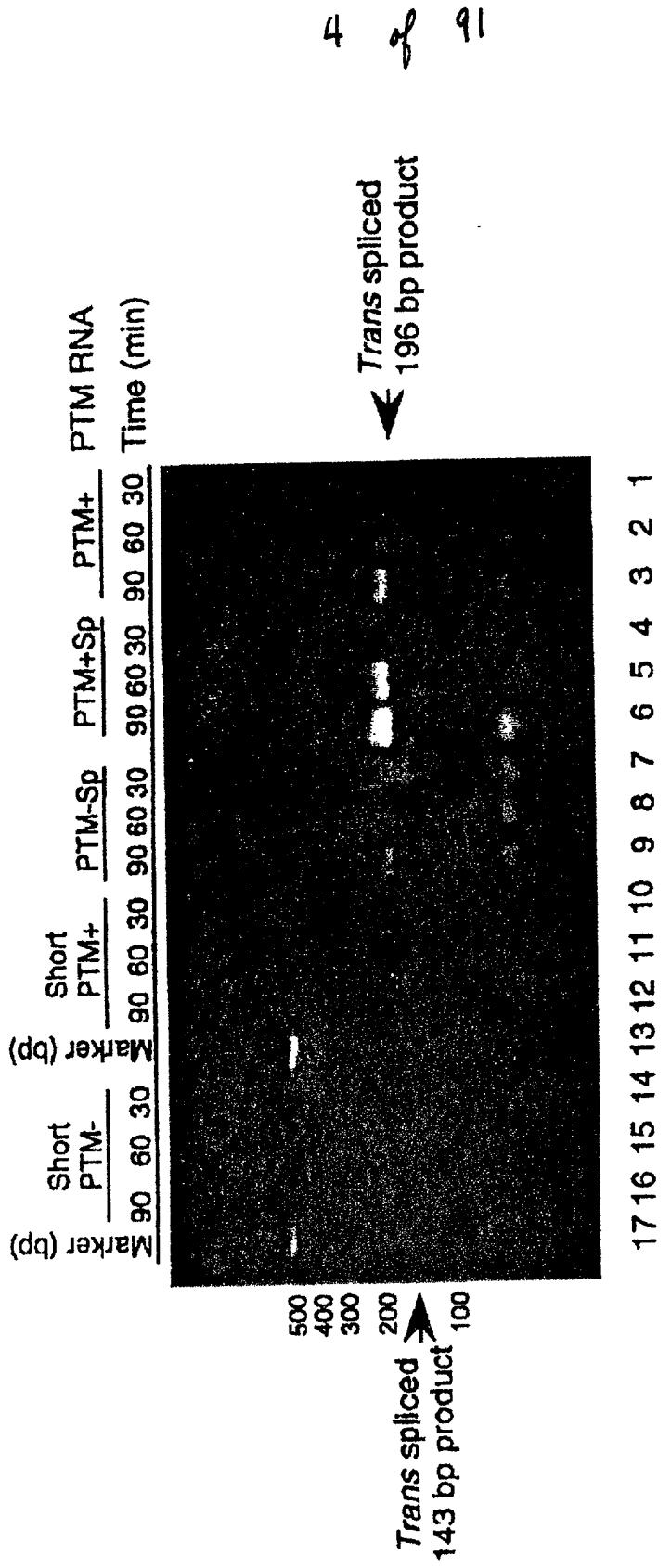


FIG. 2B

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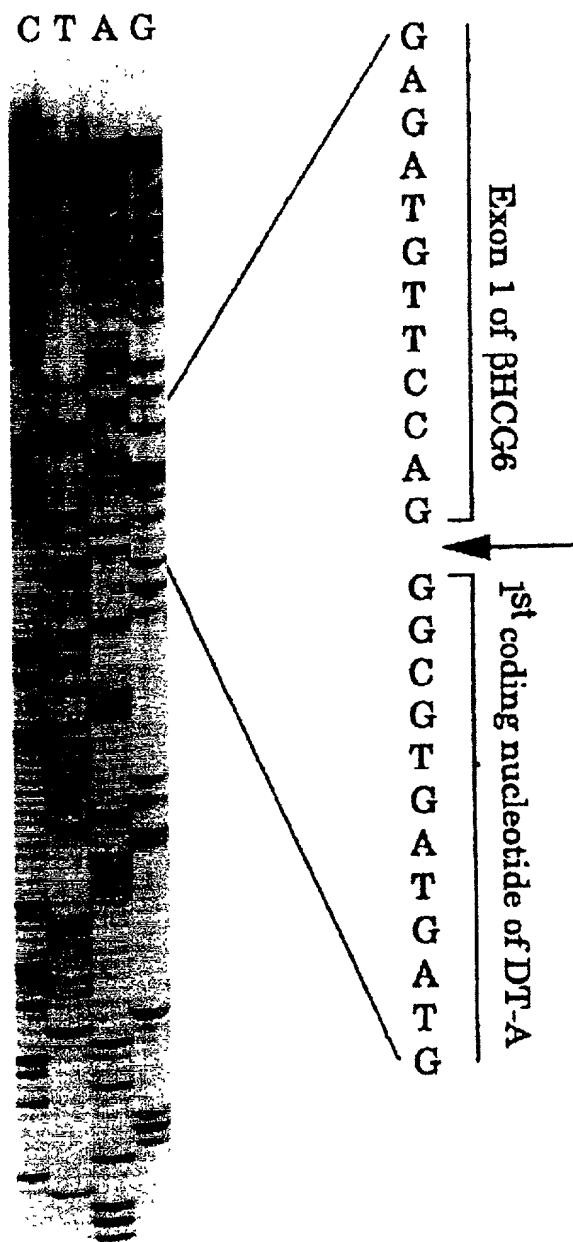
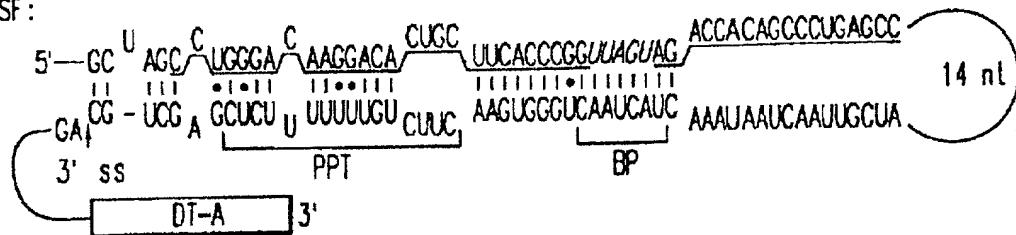


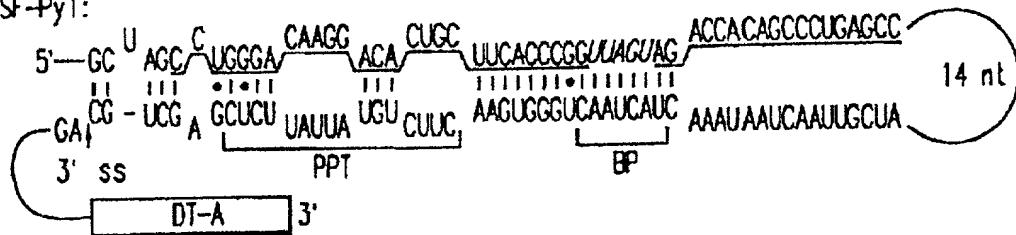
FIG.3

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1.PTM+SF:



2.PTM+SF-Py1:



3.PTM+SF-Py2:

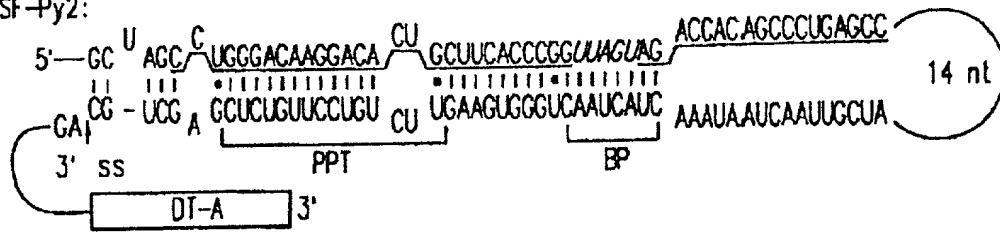


FIG.4A

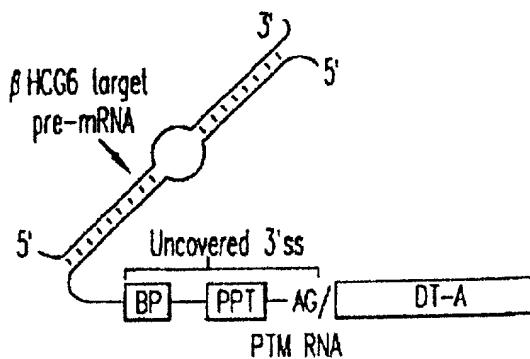


FIG.4B

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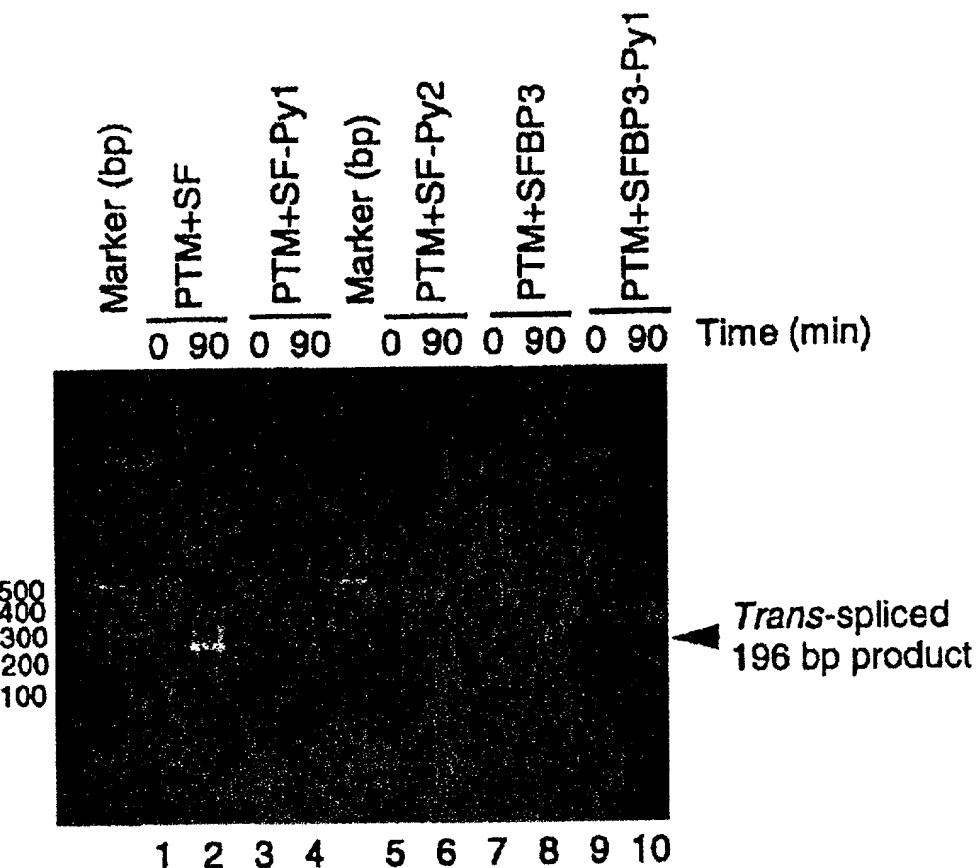


FIG.4C

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Linear PTM		Safety PTM	
Marker (bp)	βHCG-F	Marker (bp)	β-globin-F
HCGR2	DT-3R	β-globin-R	β-globin-F
DT-3R	β-globin-R	β-globin-F	βHCG-F
HCGR2	DT-3R	β-globin-R	β-globin-F
Marker (bp)	Marker (bp)	Marker (bp)	Marker (bp)

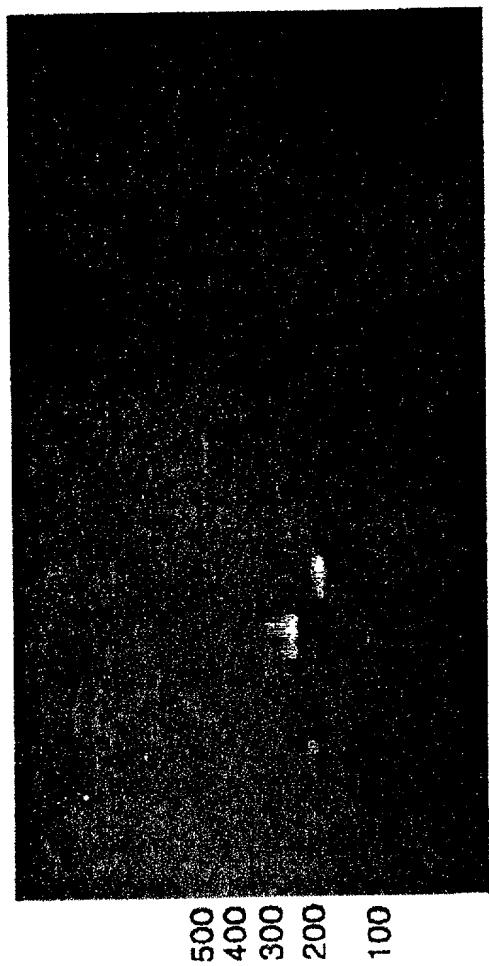


FIG. 5

FIG. 6A

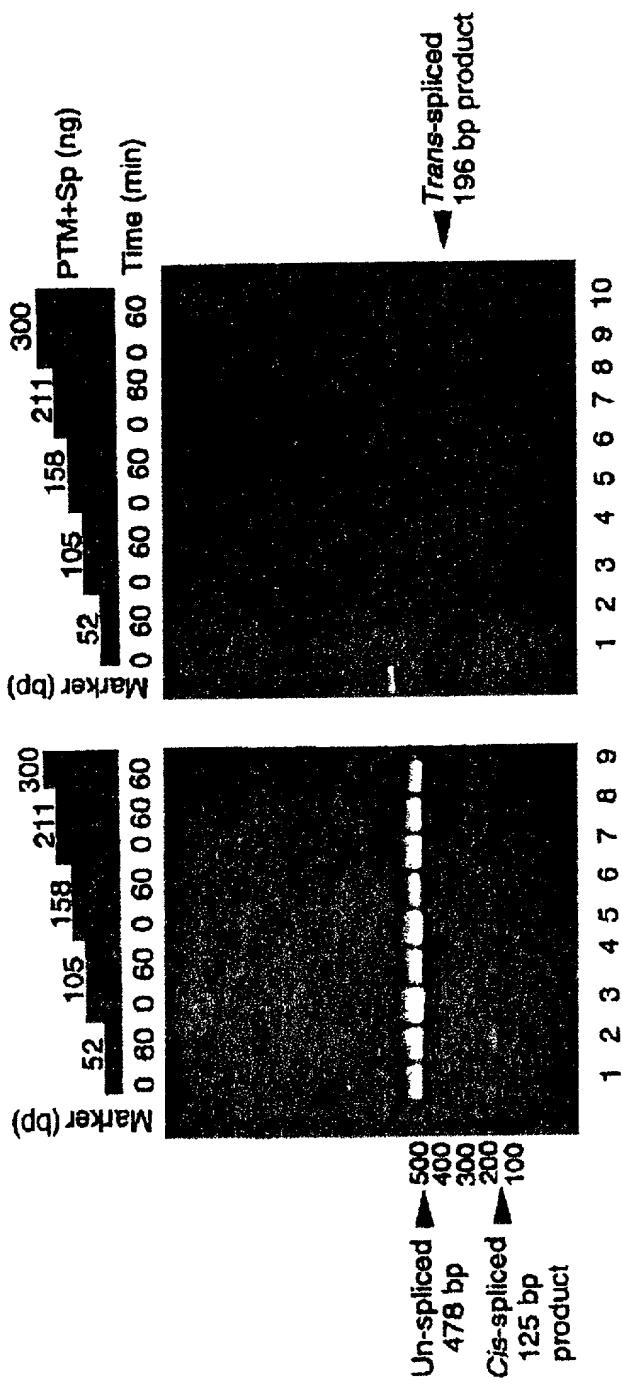


FIG. 6B

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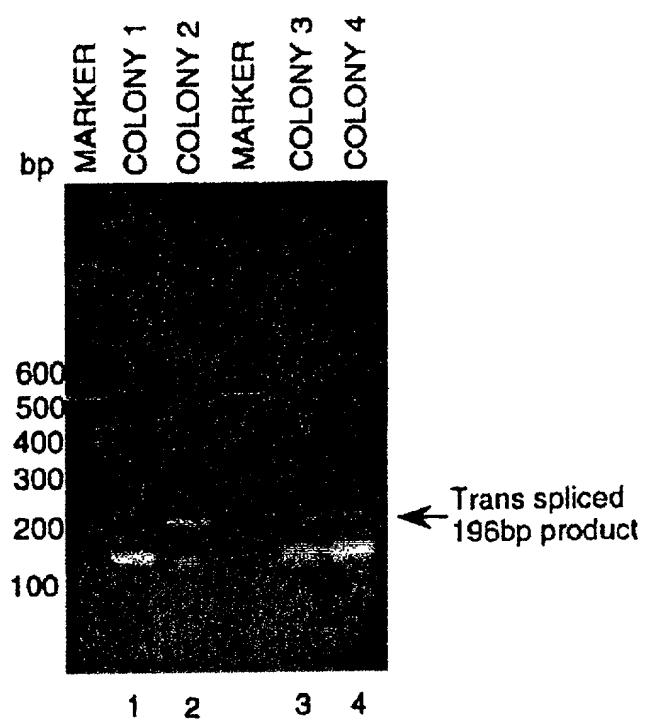


FIG. 7A

= 89

EXON 1 OF β HCG6 |
5'-CAGGGACGCCAAGGATGGAGATGTTCCAG-GGGCTGATGATGTTGTT
| 1ST CODING NUCLEOTIDE OF DT-A
GATTCTCTAAATCTTTGTGATGGAAAACCTTTTCTTCGTACACGGGACTA
AACCTGGTTATGTAGATTCATTCAAAAA-3'

FIG. 7B

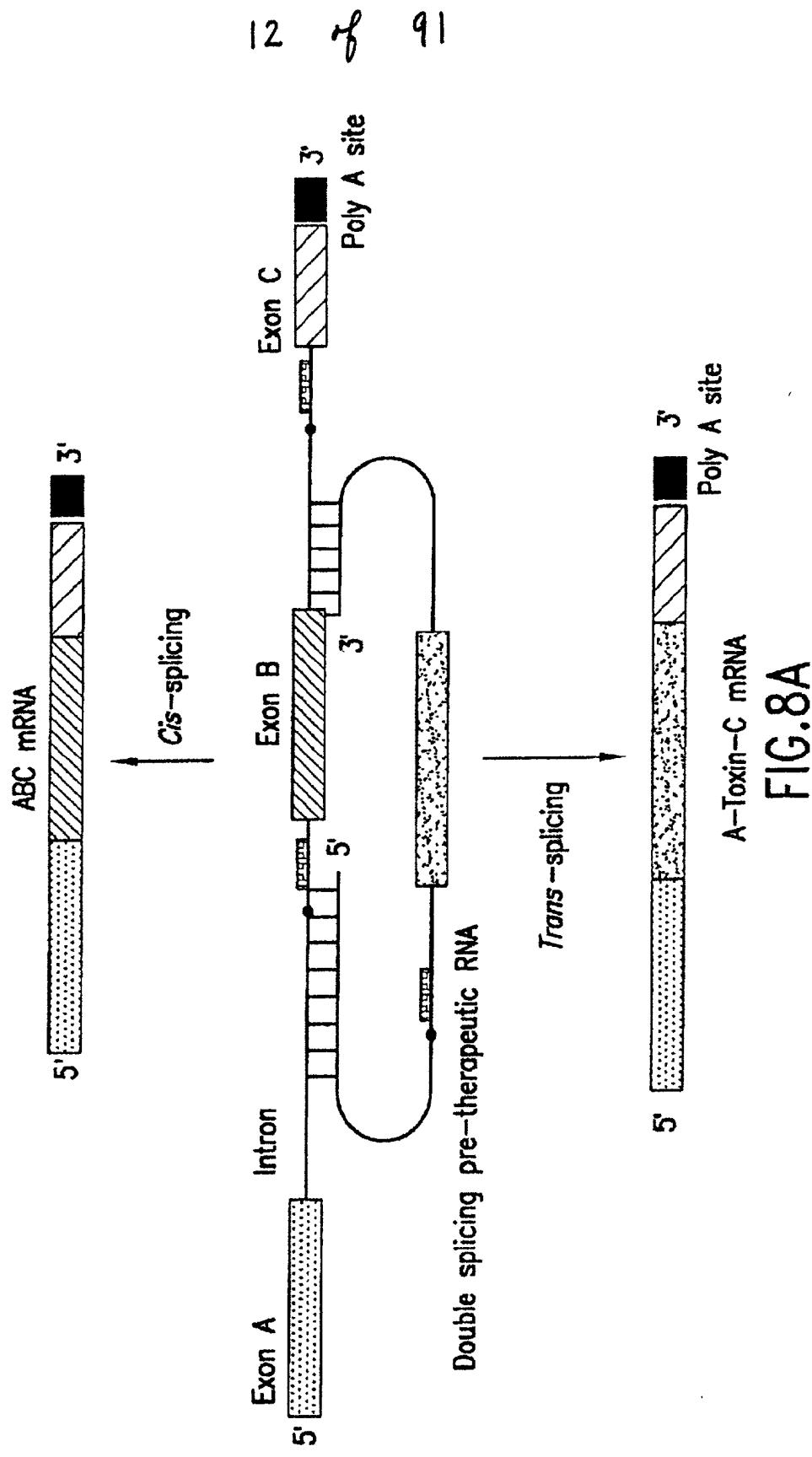
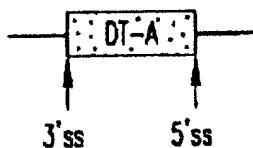
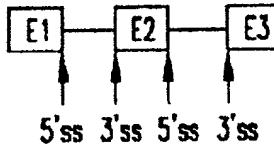
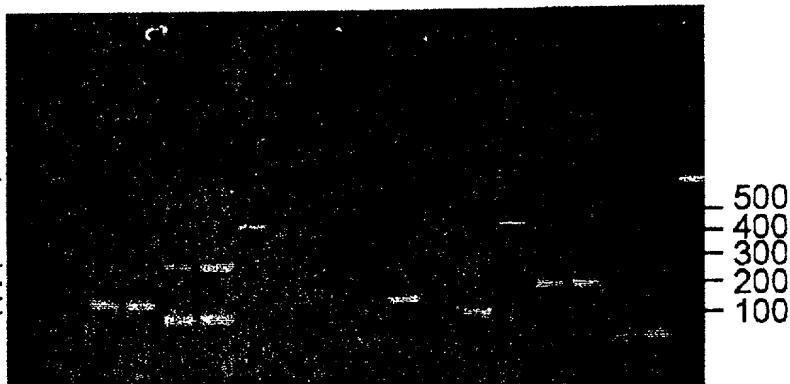
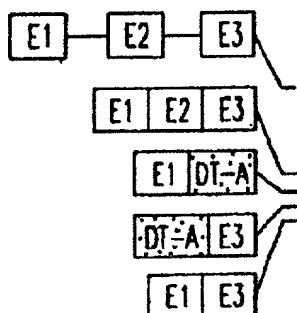
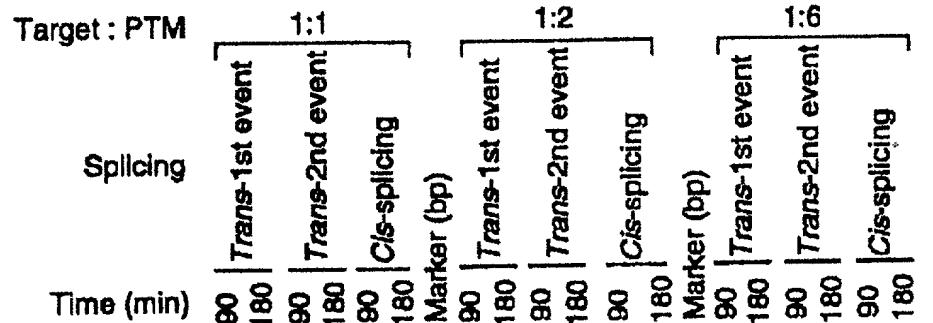


FIG.8A

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Cis-spliced products

E1 E2 E3 = NORMAL *cis*-SPLICING (277bp)

E1 E3 = Exon SKIPPING (110bp)

Trans-spliced products

E1 DT-A = 1st EVENT, 196bp. Trans-SPLICING BETWEEN 5' ss OF TARGET & 3' ss OF PTM.

[DT-A] E3 = 2nd EVENT, 161bp. Trans- SPLICING BETWEEN 3' ss OF TARGET & 5' ss OF PTM.

FIG.8B

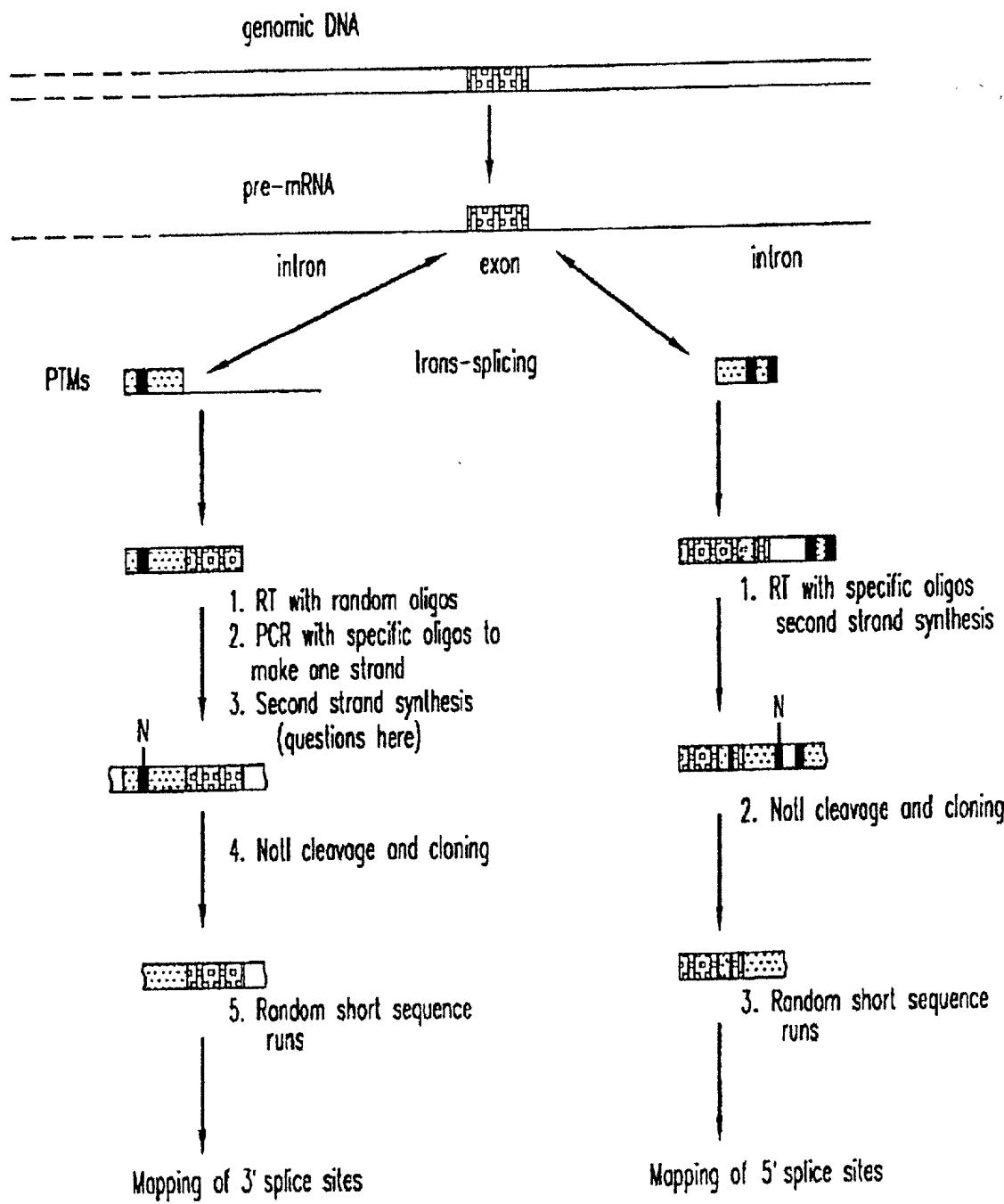


FIG.9

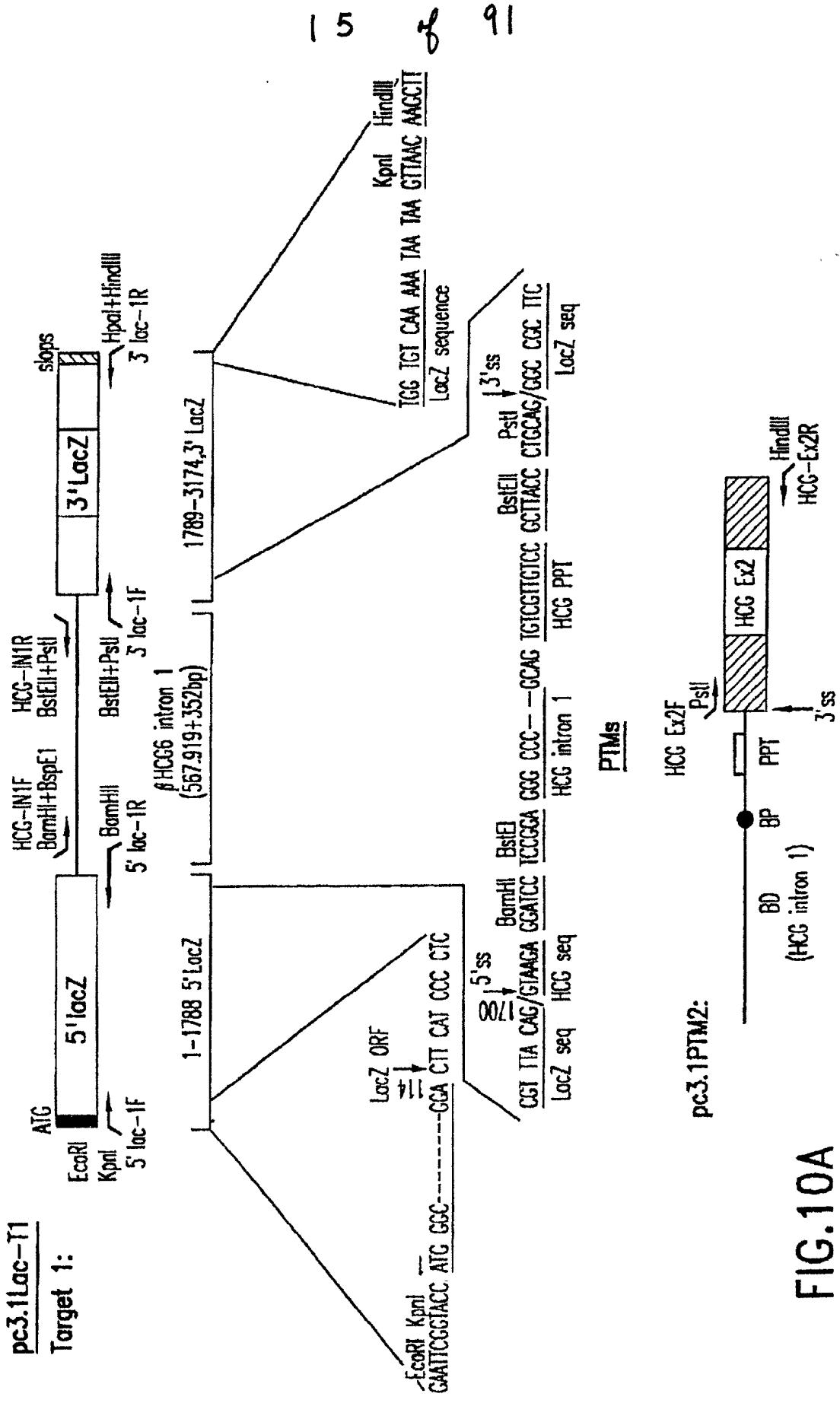


FIG. 10A

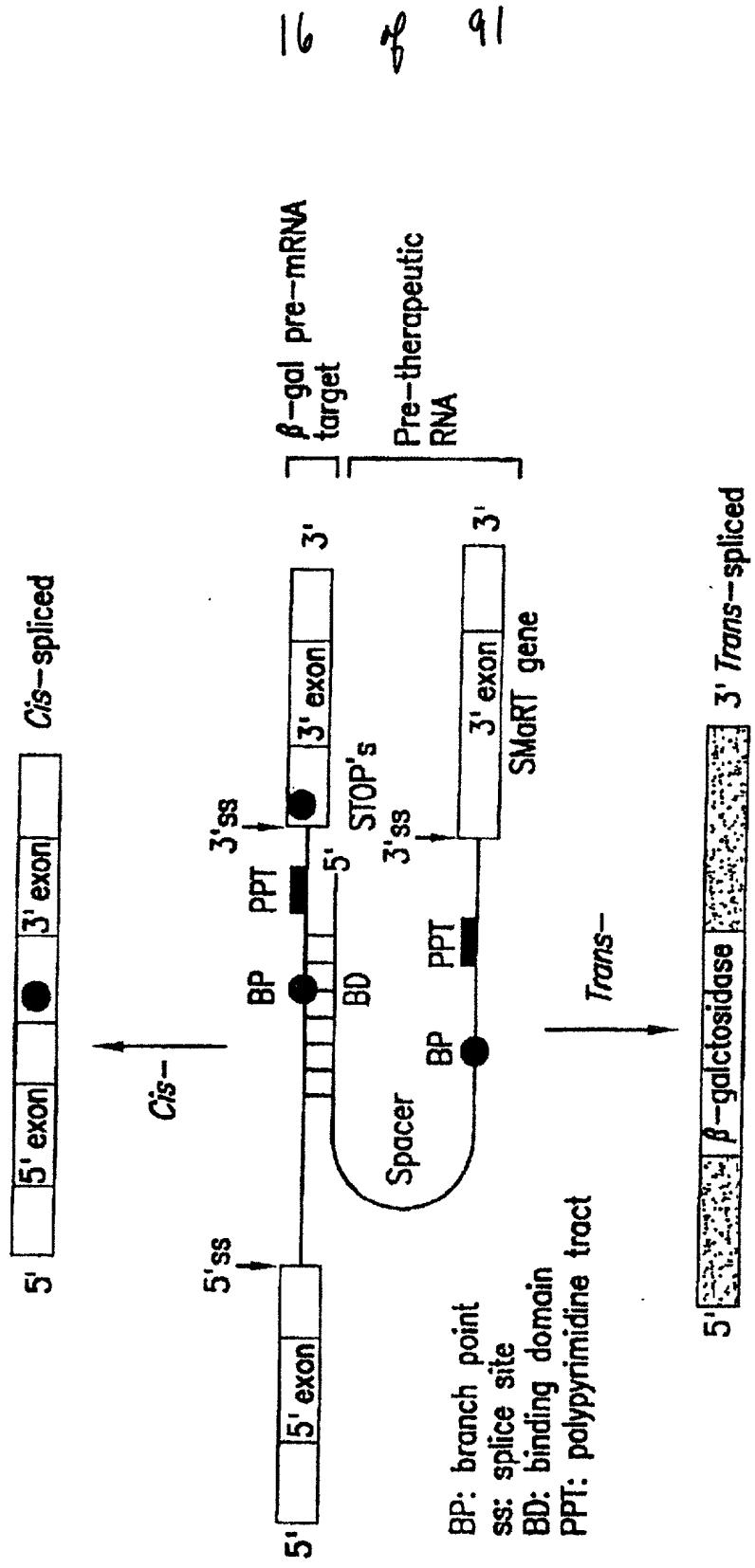


FIG. 10B

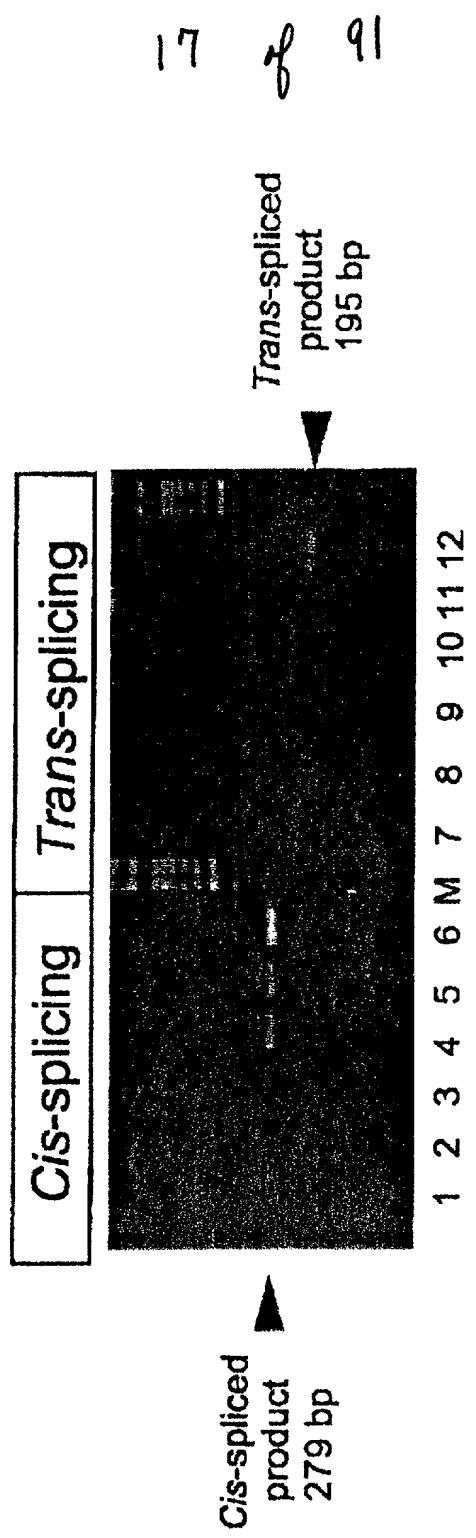


FIG. 11A

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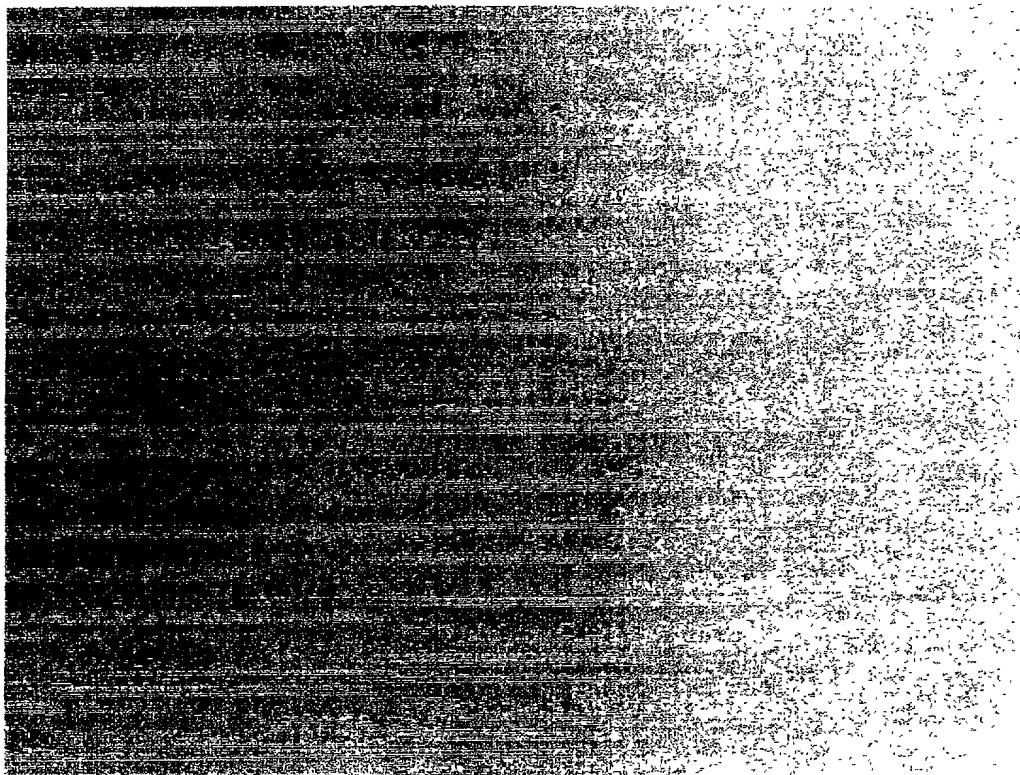


FIG.11B

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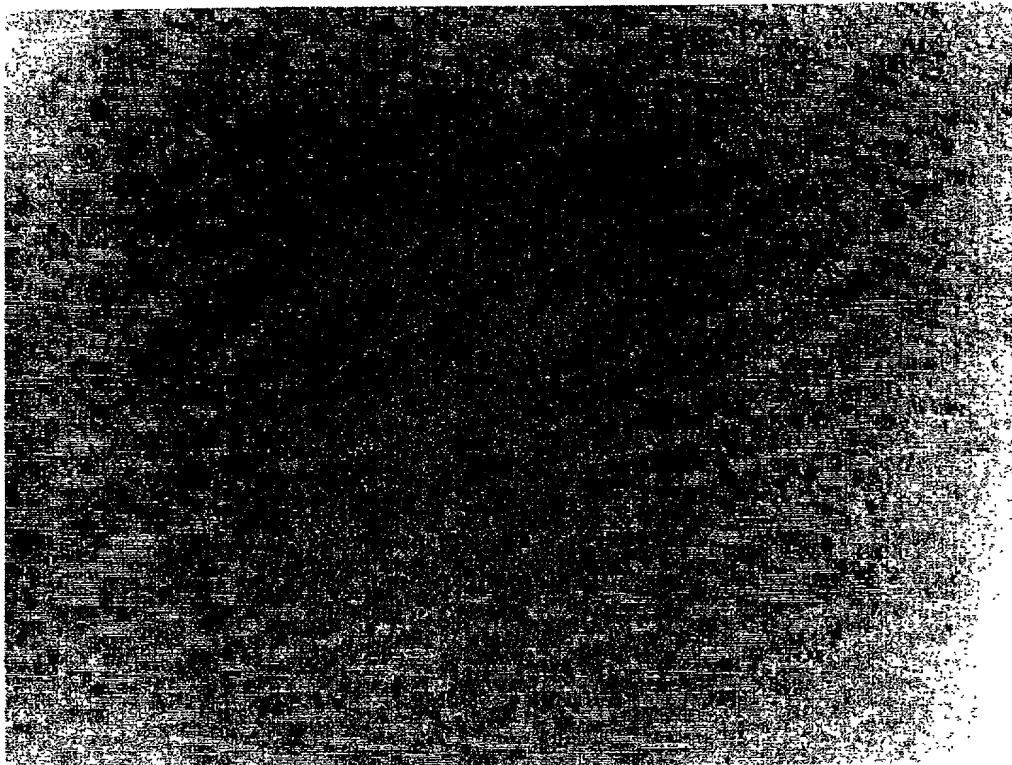


FIG.11C

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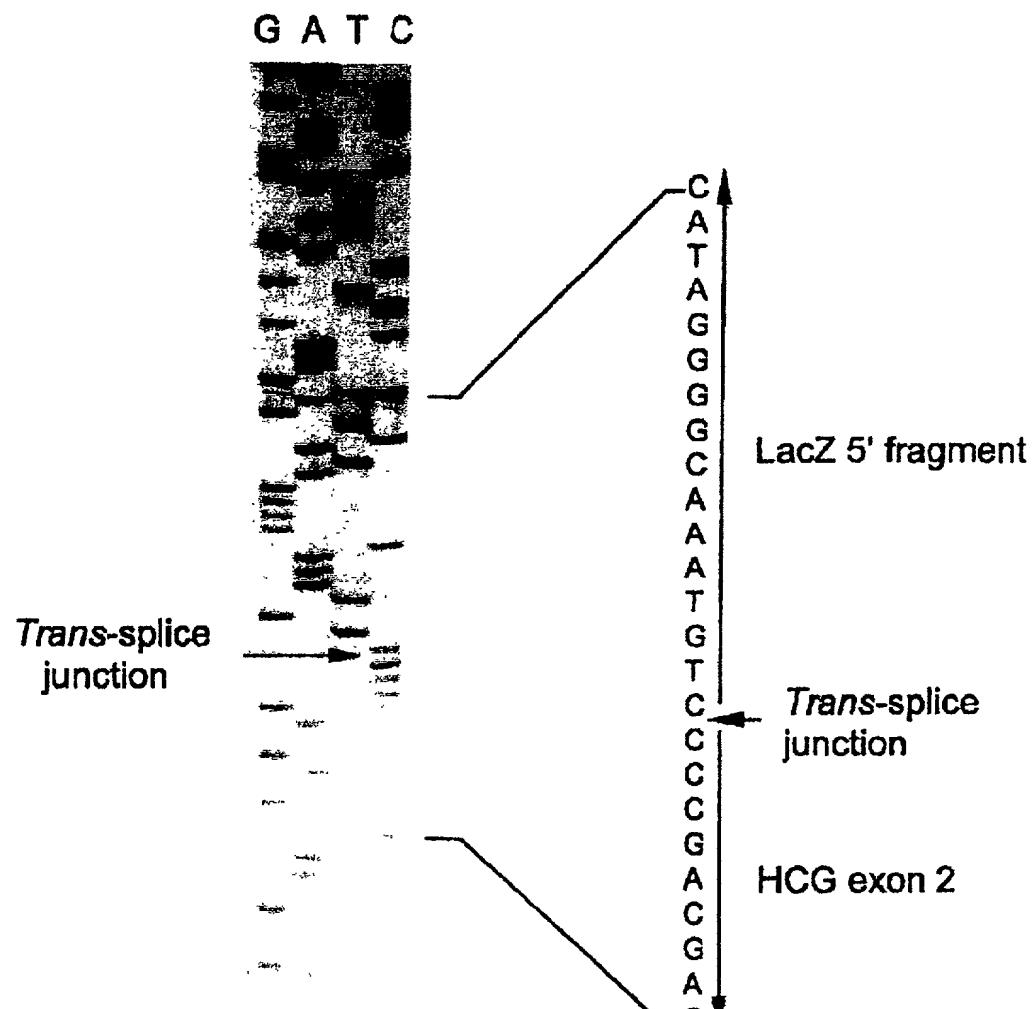


FIG.12A

1. NUCLEOTIDE SEQUENCES OF THE *cis*-SPLICED PRODUCT (285 bp):

BioLac-TR1

GGCTTTCGCTACCTCGAGAGACGGCCCGCTGATCCTTTCGAATTACACAGTCTTG

Splice junction

CGCTTTGGCTAAATACTGGCAGGGTTTCACTGATTCAGTATCCCCGTTAACG/GGCCCTTCGCTCTAATAATG

GCACTGGCTGGATCAGTCGGCTGATTAAATAATGATAAACGGCAACCCGTTGGTCGGCTTACGGGGTGATT

TGGCGATAGCCGAACGATGCCAGTTCTGTATGAAACGGTCTGGCTCTTGGCACCGCAACCGCATCCAG

2. NUCLEOTIDE SEQUENCES OF THE *trans*-SPLICED PRODUCT (195 bp)

BioLac-TR1

GGCTTTCGCTACCTGGAGAGACGGCCCGCTGATCCTTTCGAATTACACAGTCTTG

Splice junction

CGCTTTGGCTAAATACTGGCAGGGCTTTCGTCAGTATCCCCTTACAG/GGCCCTGCTCTTGGCTGCT

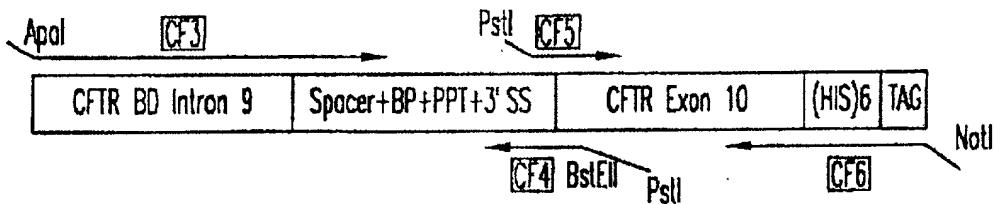
HCCR2

GAGCATGGCGGGACATGGCAATCCAAGGAGCCACTTGGCCACGGTGGCG

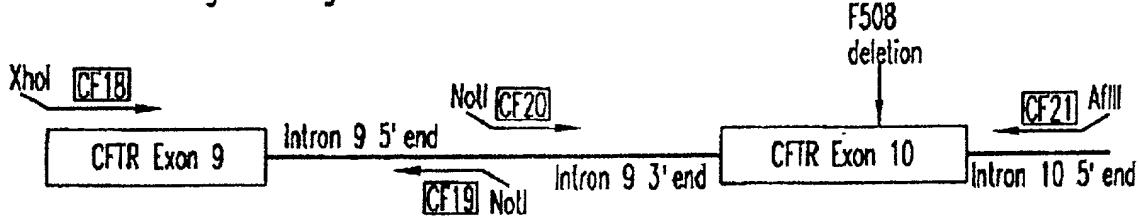
FIG. 12B

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CFTR Pre-therapeutic molecule (PTM or "bullet")



CFTR mini-gene target-construction



Trans-splicing Repair

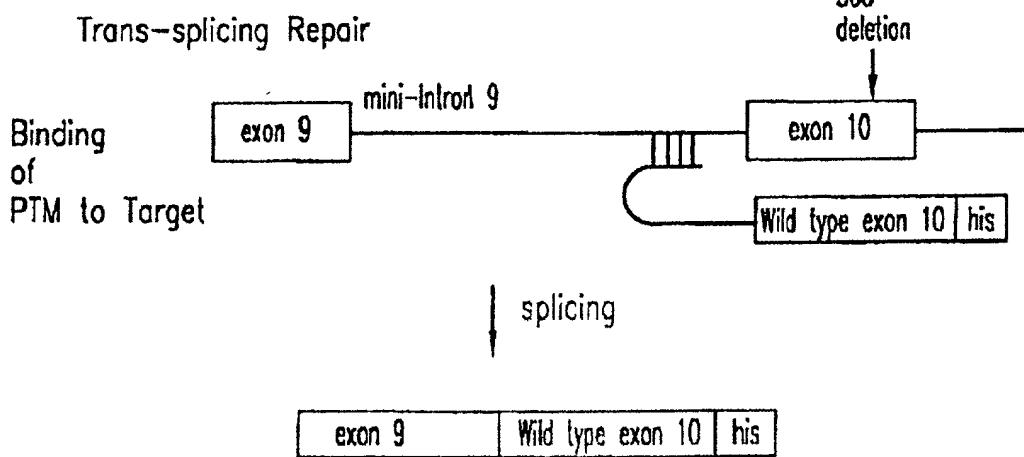


FIG.13

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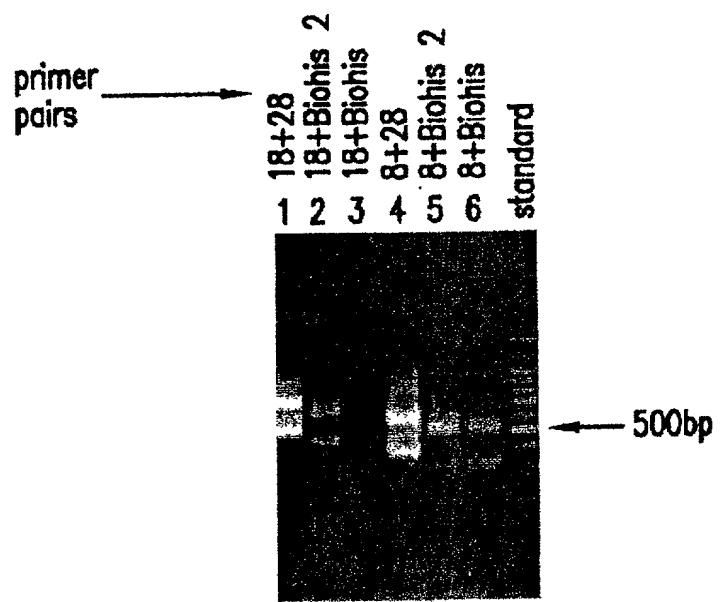


FIG.14

DNA sequence 500 b.p.: GCTAGCGTTAA ... TGGCCACTCCAC linear

Positions of Restriction Endonucleases sites (unique sites underlined)

The diagram illustrates the structure of the c-fos gene, highlighting various restriction enzyme cleavage sites and the location of the binding domain relative to the exon-intron boundaries.

Restriction Enzyme Sites:

- Dra I:** Located in the 5' flanking region at position 8.
- Xba I:** Located in the 5' flanking region at position 15.
- Ban I:** Located in the 5' flanking region at position 15.
- Apa I:** Located in the first intron (INTRON 9 BD) at position 16.
- Sac I:** Located in the first intron (INTRON 9 BD) at position 16.
- Nhe I:** Located in the first intron (INTRON 9 BD) at position 16.
- Pst I:** Located in the 3' flanking region at position 102.
- Xmn I:** Located in the 3' flanking region at position 172.
- Dde I:** Located in the 3' flanking region at position 190.
- Sph I:** Located in the 3' flanking region at position 282.
- Xba I:** Located in the 3' flanking region at position 282.

Exons and Introns:

- Exon 1:** Coding sequence from position 1 to 44.
- Intron 9 BD:** Non-coding sequence between positions 16 and 72.
- Exon 10 CFTR + HIS TAG + STOP:** Coding sequence from position 72 to 282, ending with a stop codon.
- Exon 11:** Coding sequence from position 282 to 324.

Binding Domain: The binding domain is located within the first intron (INTRON 9 BD), spanning from position 16 to 72. It is indicated by a bracket above the sequence.

FIG. 15A

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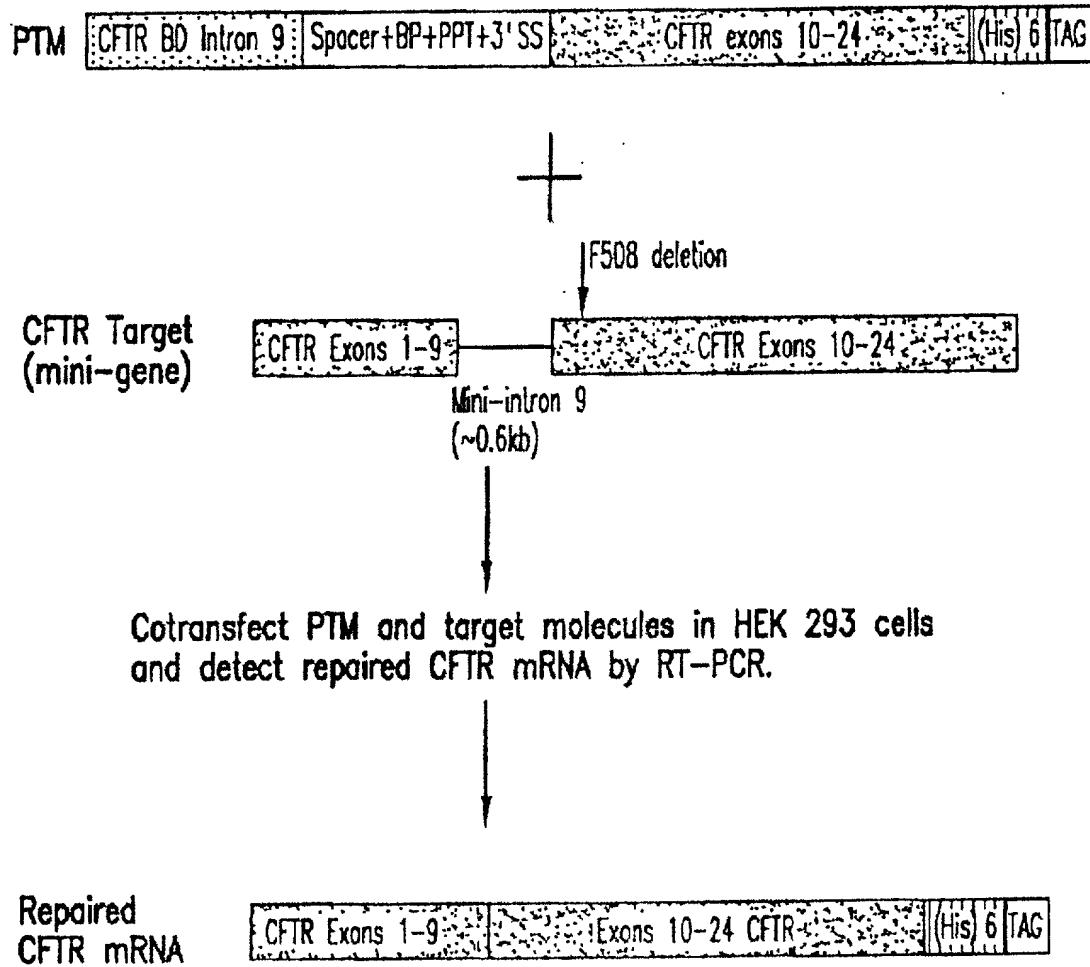


FIG.16

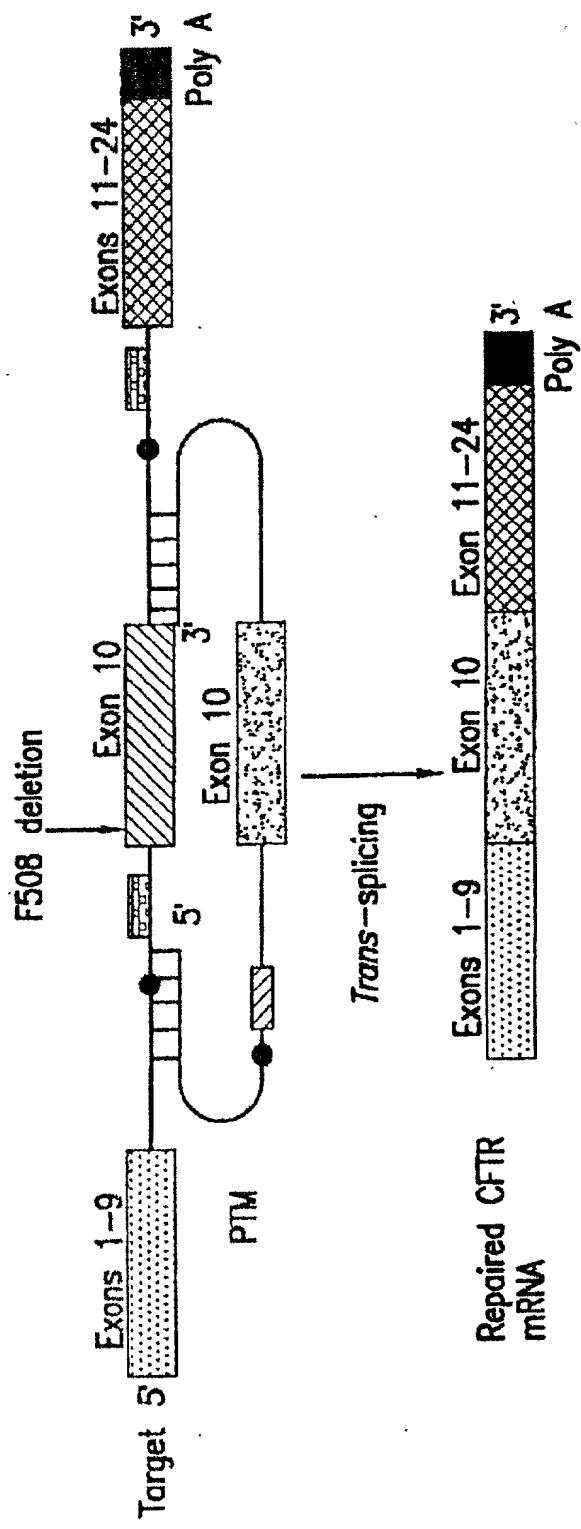
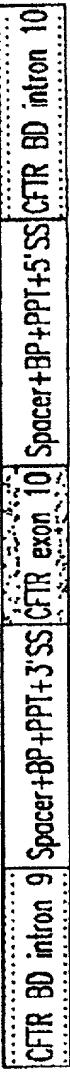


FIG.17

DOUBLE TRANS-SPlicing SPECIFIC TARGET

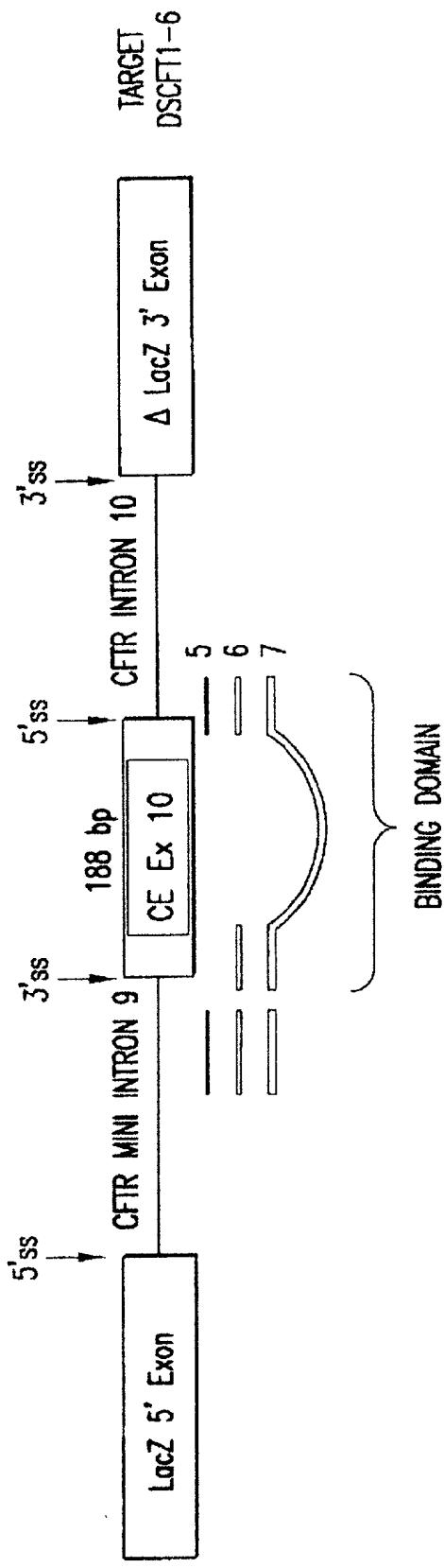


FIG. 18

DOUBLE TRANS-SPlicing PTMs

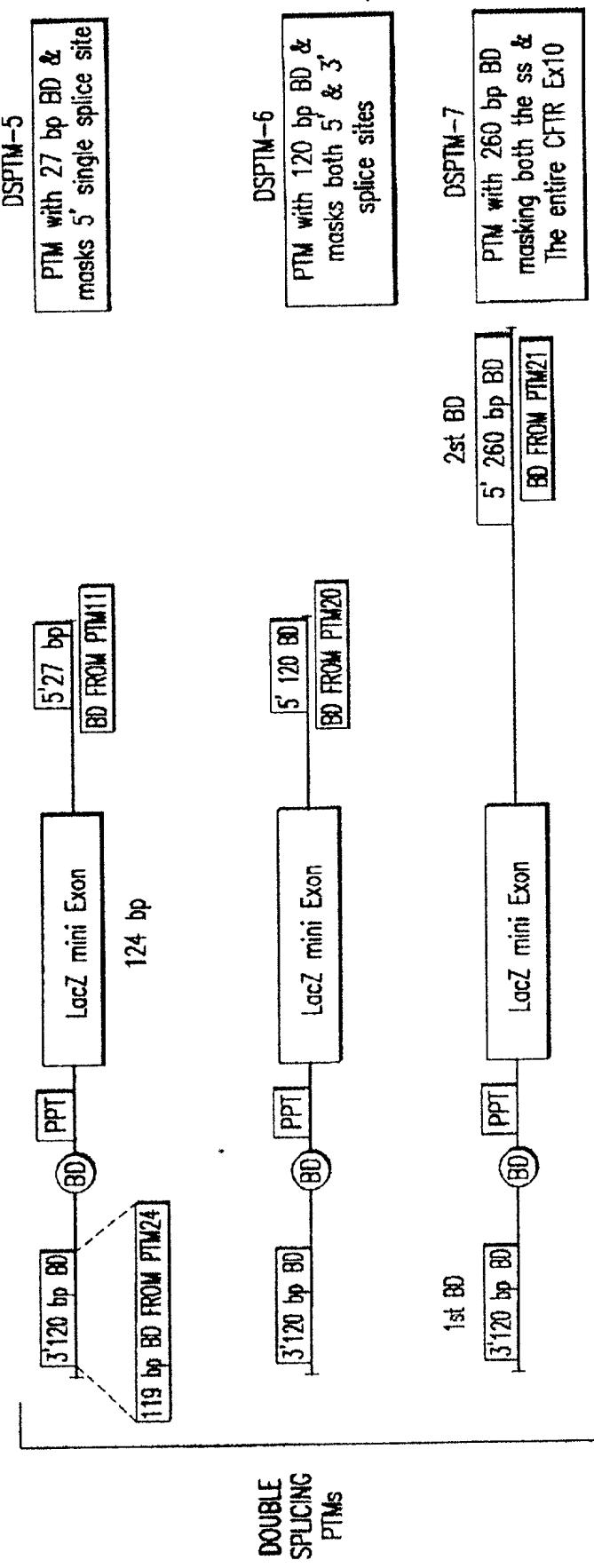


FIG. 19

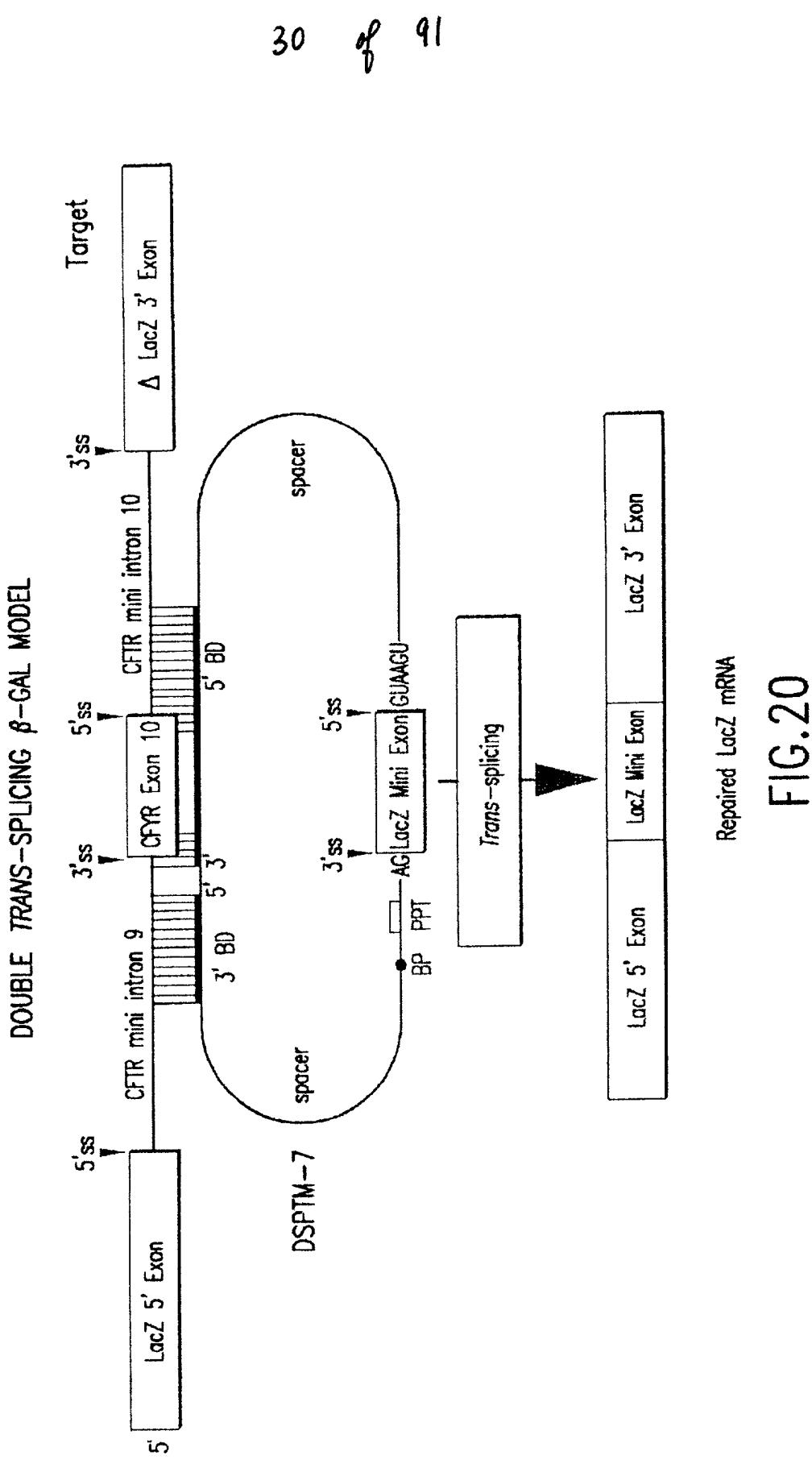
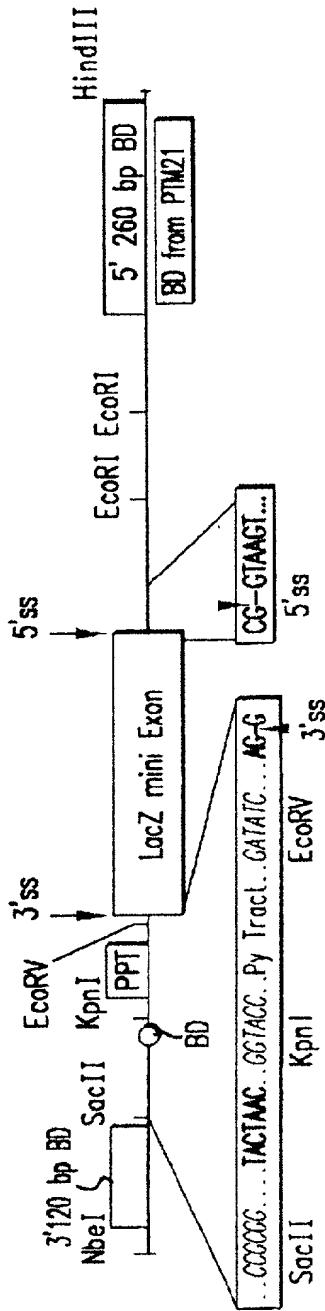


FIG.20



(1) 3' BD (120 BP): CATTCACTTGCCTCAATTATCATCTAACCGAAACTGATAATTCTTATTGTAAAGCTTATTAACTCATTGATTC
AAAAATTAAATTAACCTCCCTGTCTCATACTCTGCTATGCCAC

(2) Spacer sequences (24 bp): AACATTATTAAACGTTGGCTCAA

(3) Branch point, pyrimidine tract and acceptor splice site: TACIAAC 1 GG TACC TCTCTTTTTTTT GATA TCCTGAG ATTGGGGCCCTTCGA TACG
LacZ mini 5' ss
exon

(4) 5' donor site and 2nd spacer sequence: TGA AUG GTAAAGT GTTATCACCGATAATGTTCTAACCTGATTACACTGAACTTCAATTAAACCCATTAACTCA
CTAAGATCCACCGG

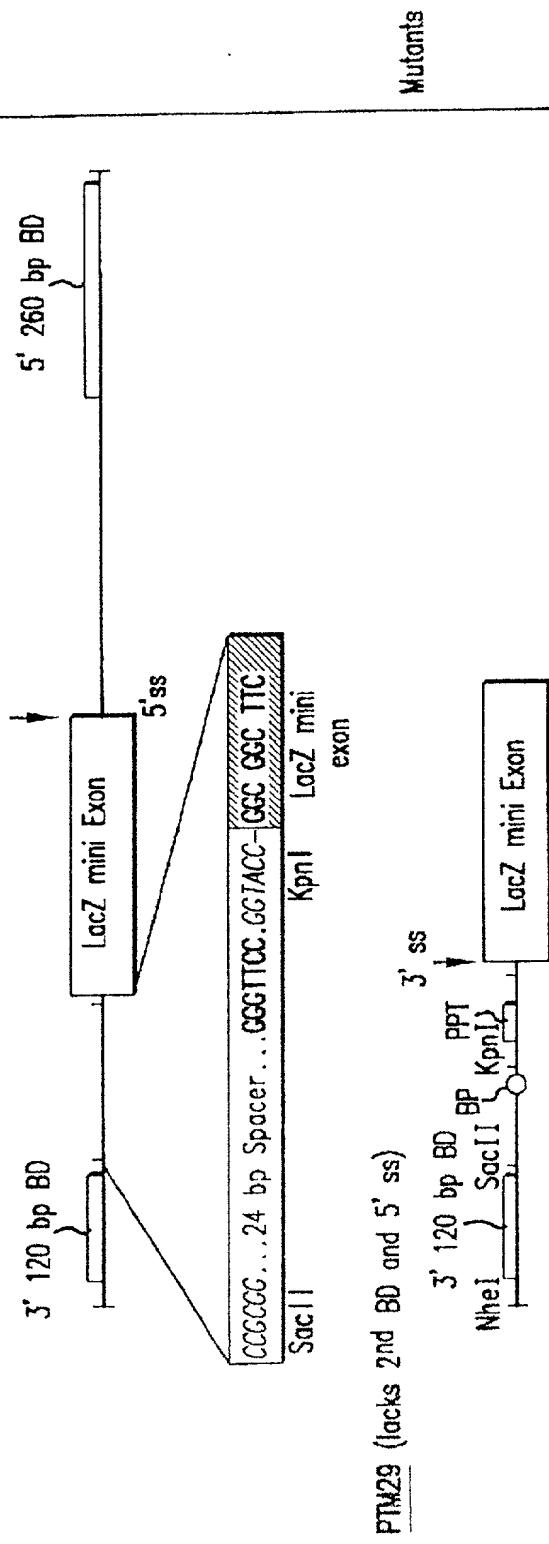
(5) 5' BD (260 BP): TCAAAAAGTTTCACATAATTCTTACCTCTCTCTGAATTCACTGCTTGTGACCC TCTCTGAACTTCAATTCAATTGCTTAA
ACACCAAATGATTTCCTTAATGGTGCTGGCATTAATCCCTGAAACTGATAACACAA TGAATTCTTCCACTGTCCTAA
AAAAACCCCTGAAATTCTCCATTTCATTACACTGAACTTCAATTAAACCCATTAACTCA
TTATCAAATCACGC

91

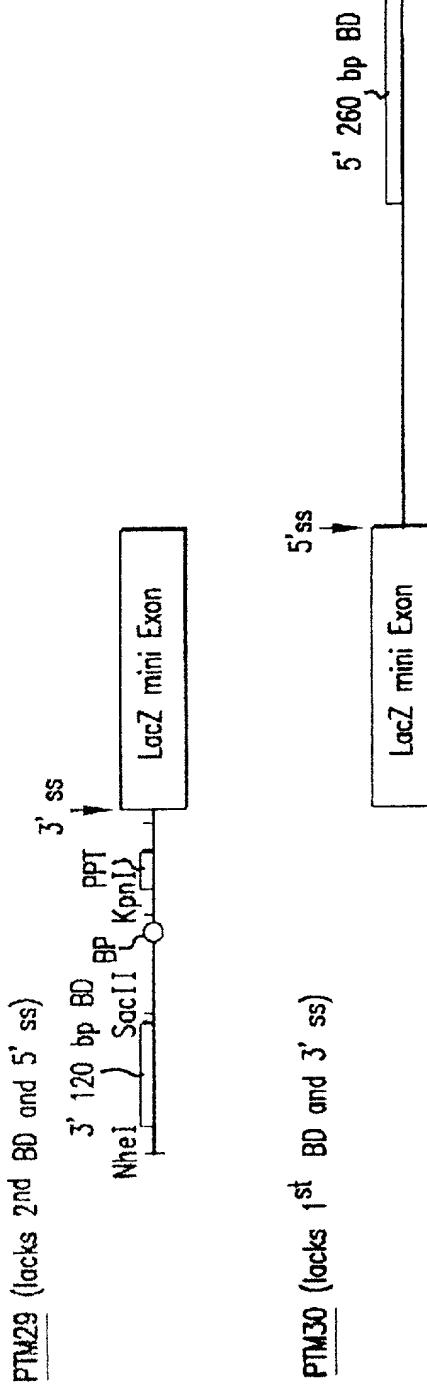
FIG. 21

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DSPTM8: (Δ 3' ss; 3' splice elements i.e. BP, PPT & AG dinucleotide has been deleted and replaced with random sequences, but still has the functional 5' splice site)



Mutants



PTM30 (lacks 1st BD and 3' ss)



FIG.22

ACCURACY OF DOUBLE TRANS-SPlicing REACTION

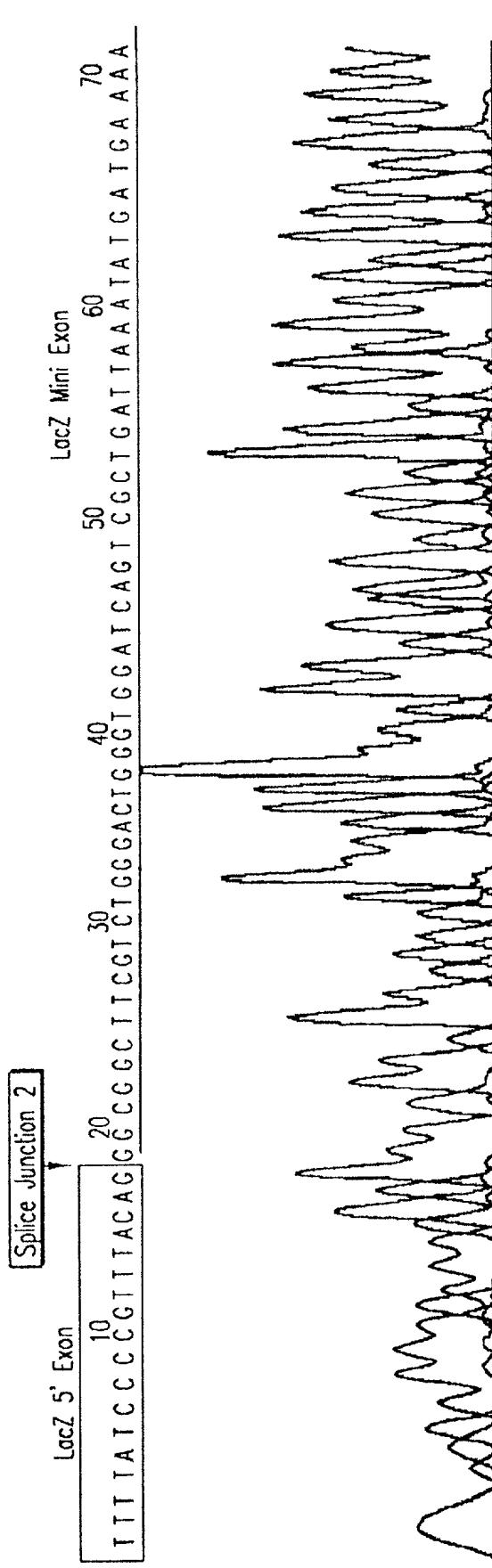


FIG. 23A

ACCURACY OF DOUBLE TRANS-SPlicing REACTION

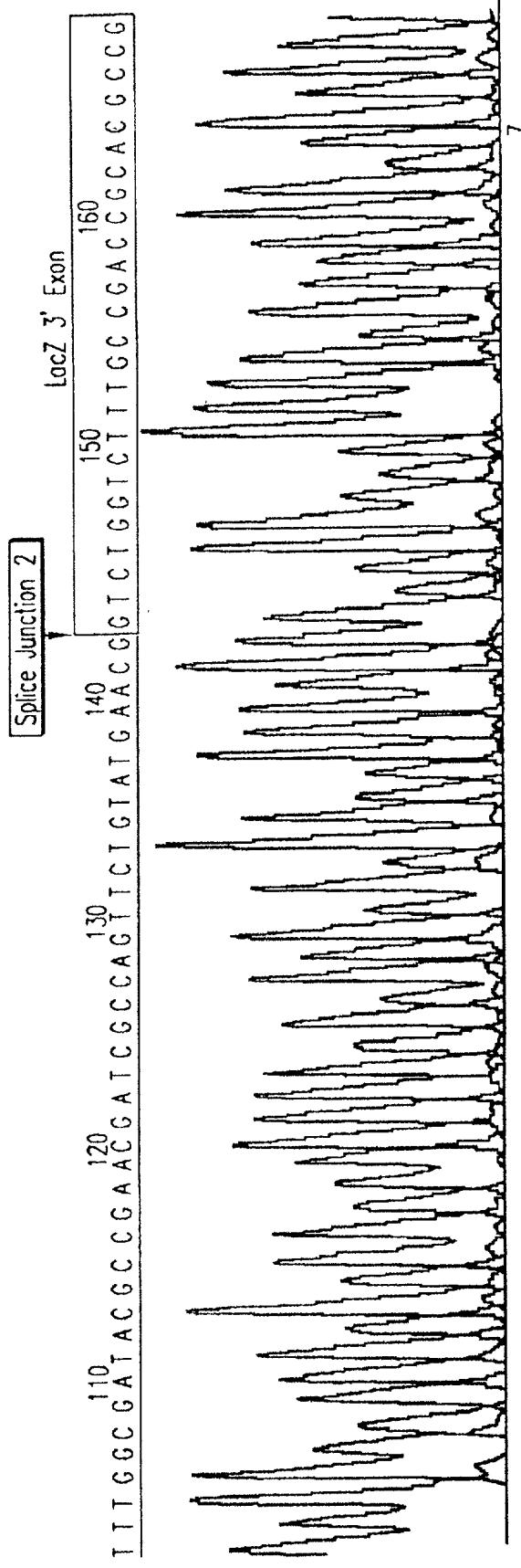
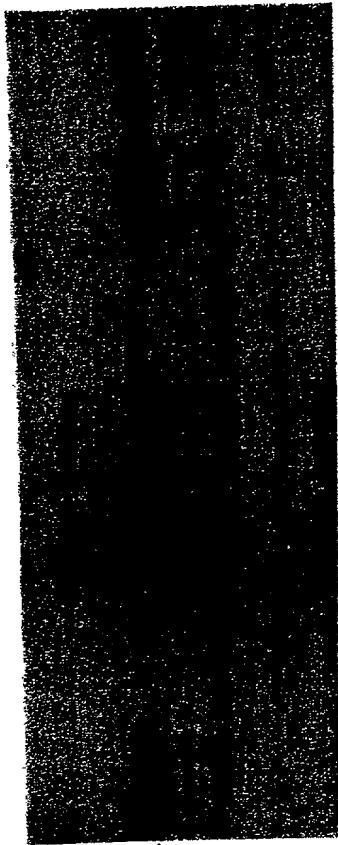
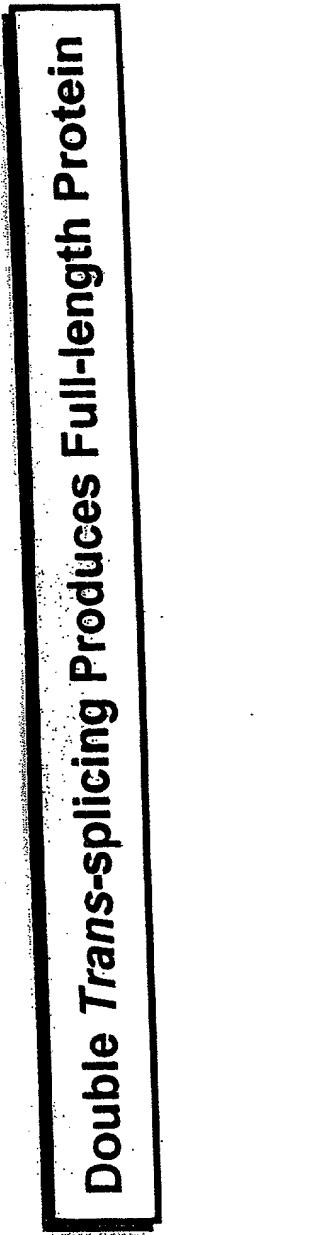


FIG.23B



1 2 3 4 5 6 7

Lane 1: DSCFTT.6 Target alone	25 µg
Lane 2: DSPTM7	25 µg
Lane 3 Target + PTM #6	25 µg
Lane 4: Target + PTM #9	25 µg
Lane 5: Delta 3' splice mutant alone	25 µg
Lane 6: Target + Delta 3' ss	25 µg
Lane 7: Target+PTM29+30 (mutants)	25 µg

Figure 24

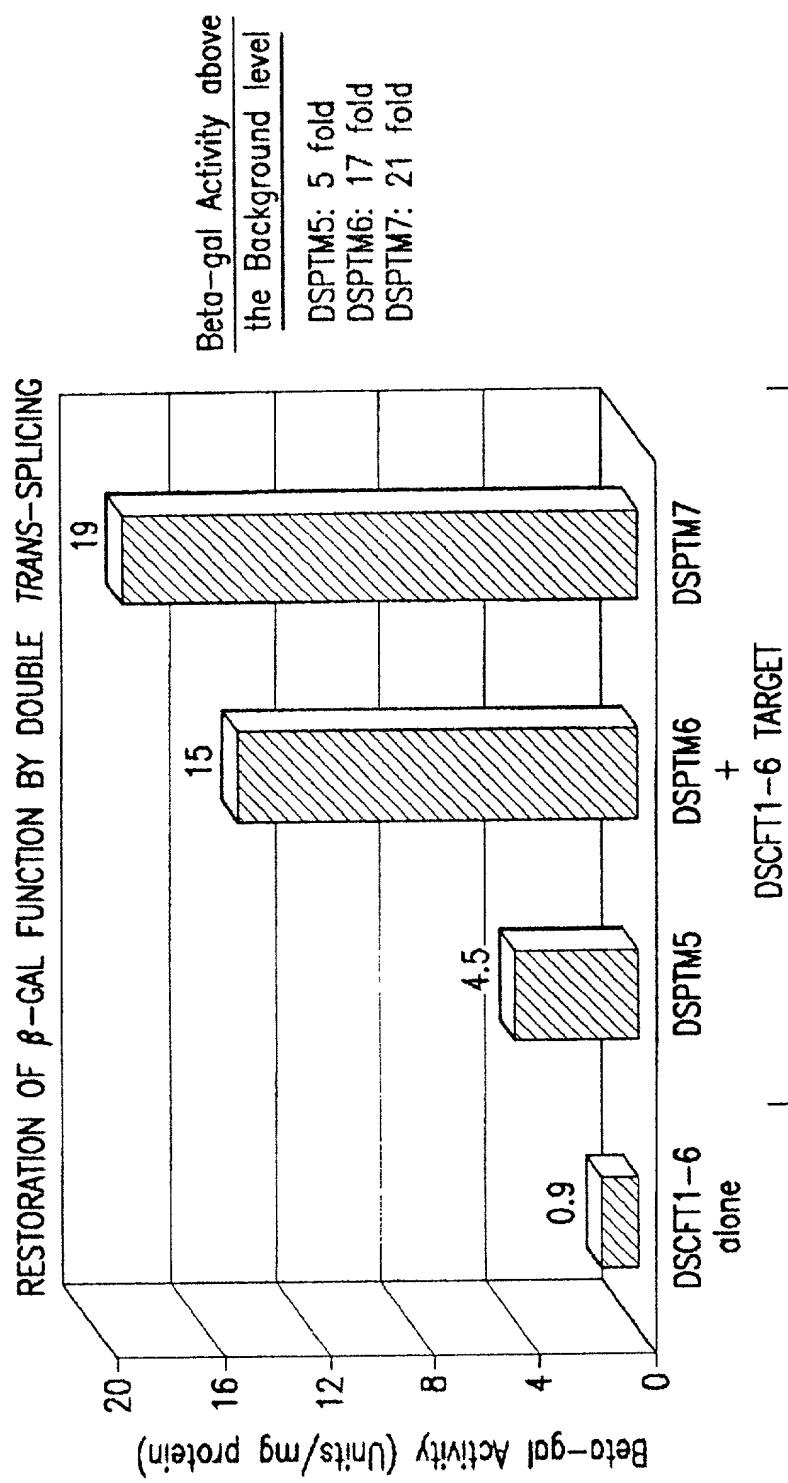


FIG. 25

RESTORATION OF β -GAL ACTIVITY IS DUE TO DOUBLE RNA
TRANS-SPLICING EVENTS

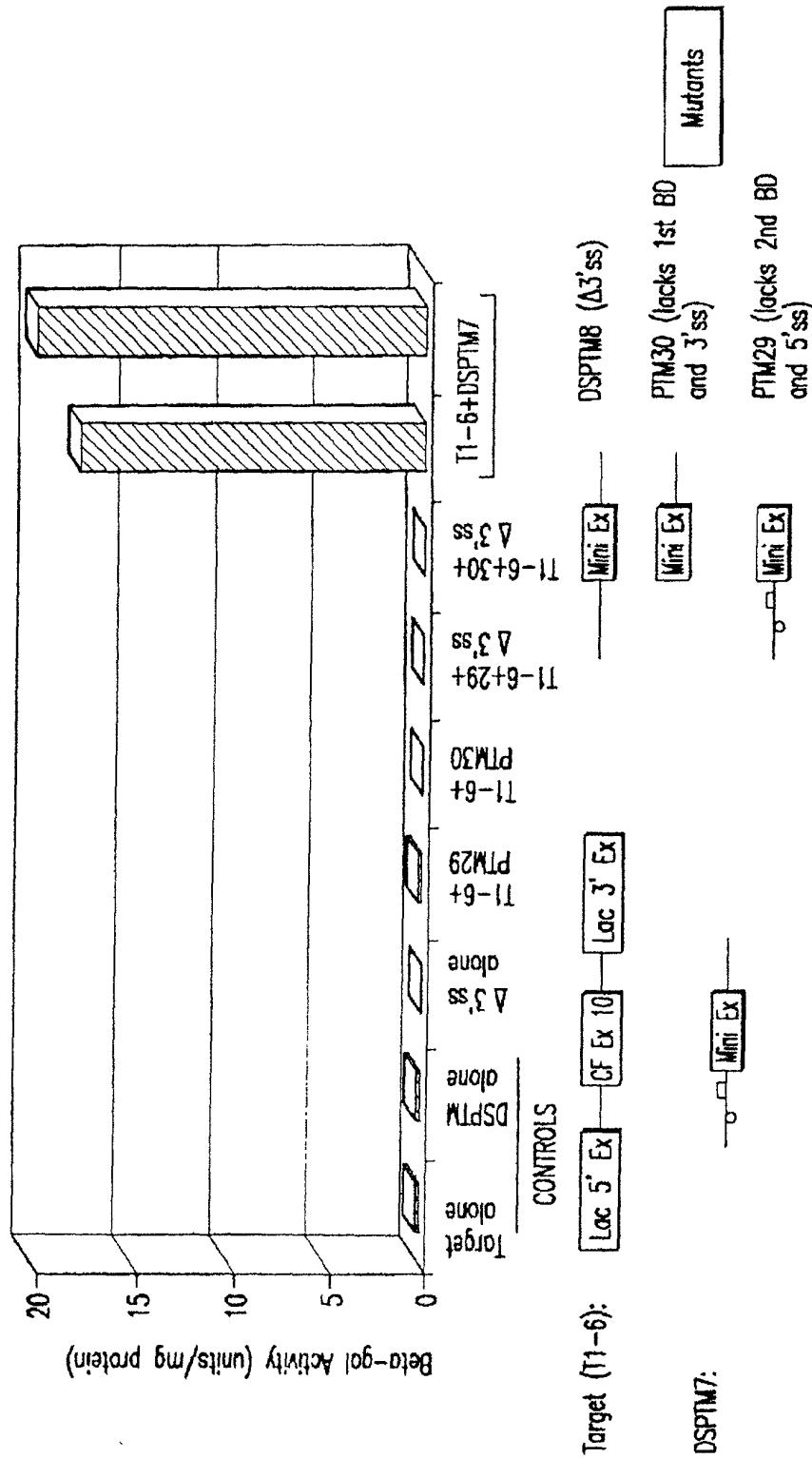


FIG. 26

DOUBLE TRANS-SPLICING: TITRATION OF TARGET & PTM

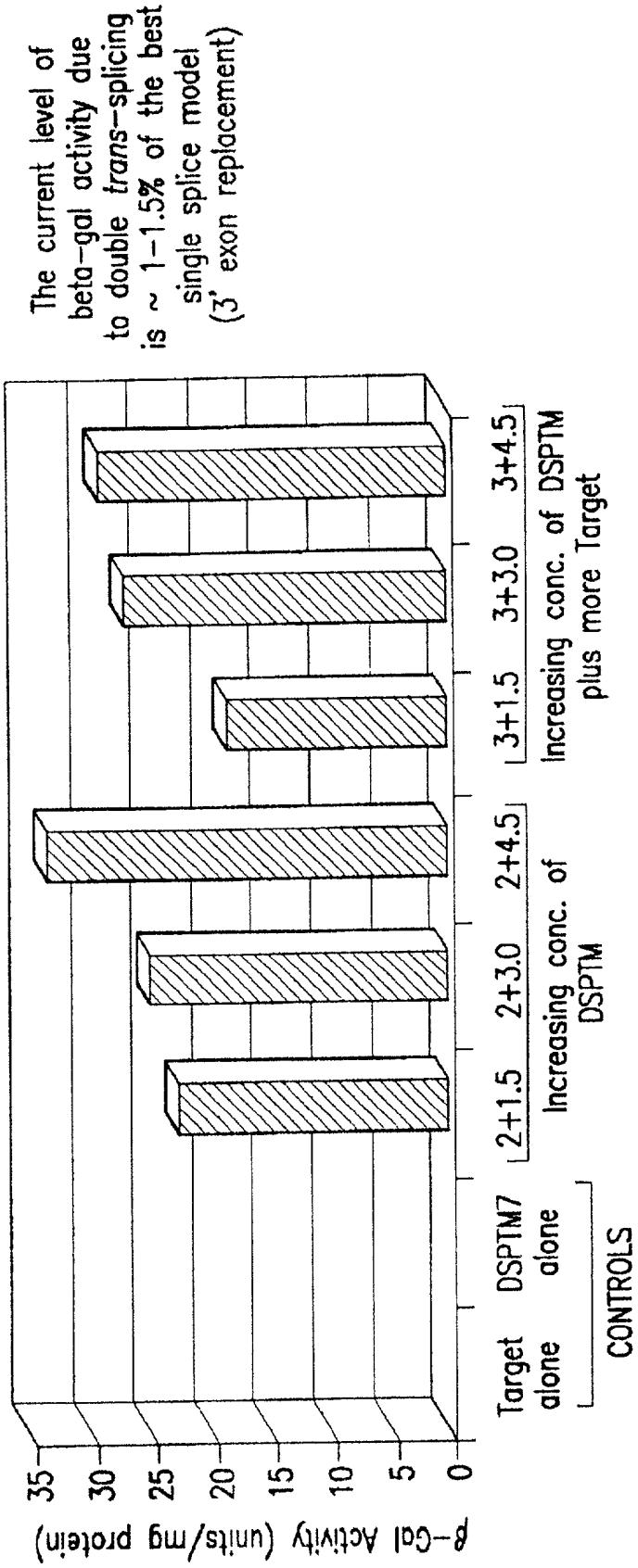


FIG. 27

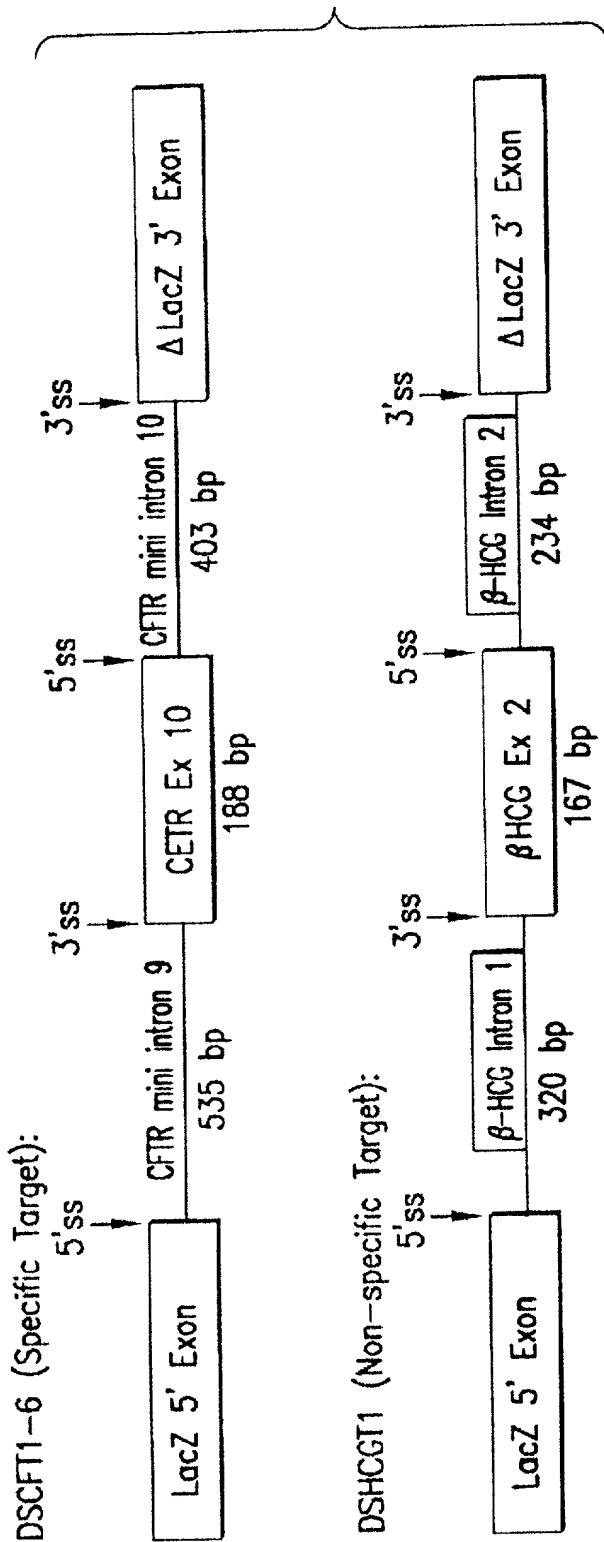


FIG. 28

SPECIFICITY OF DOUBLE TRANS-SPlicing REACTION

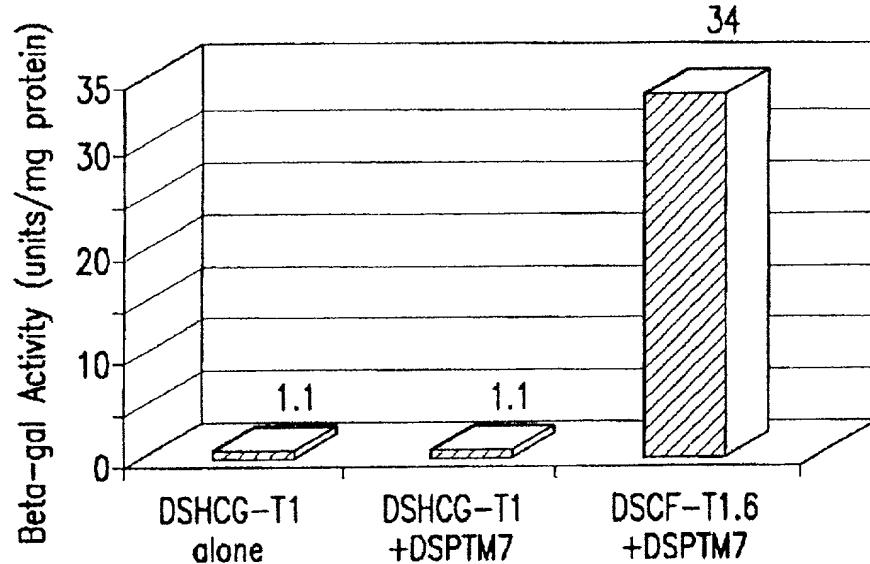


FIG.29

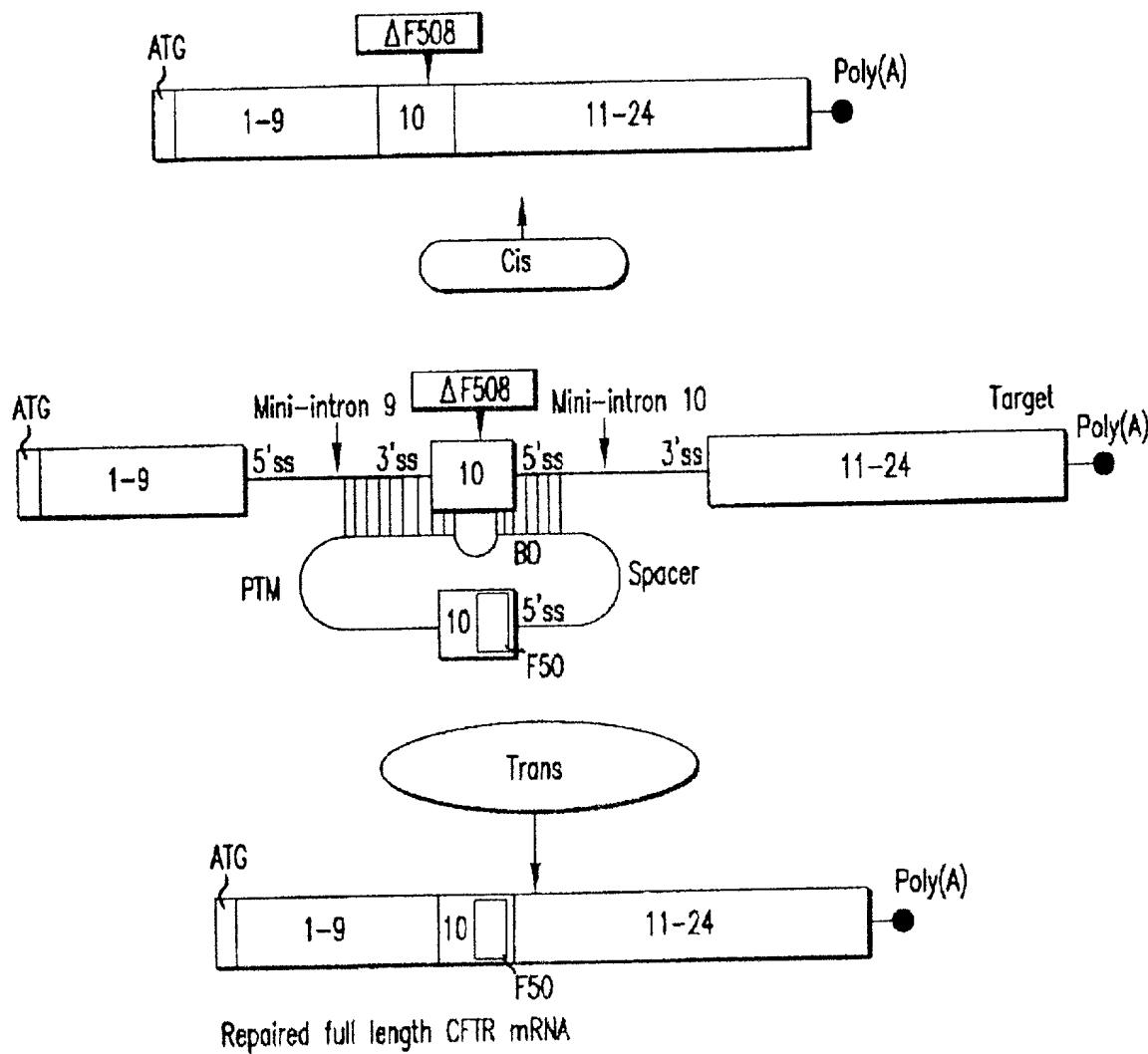
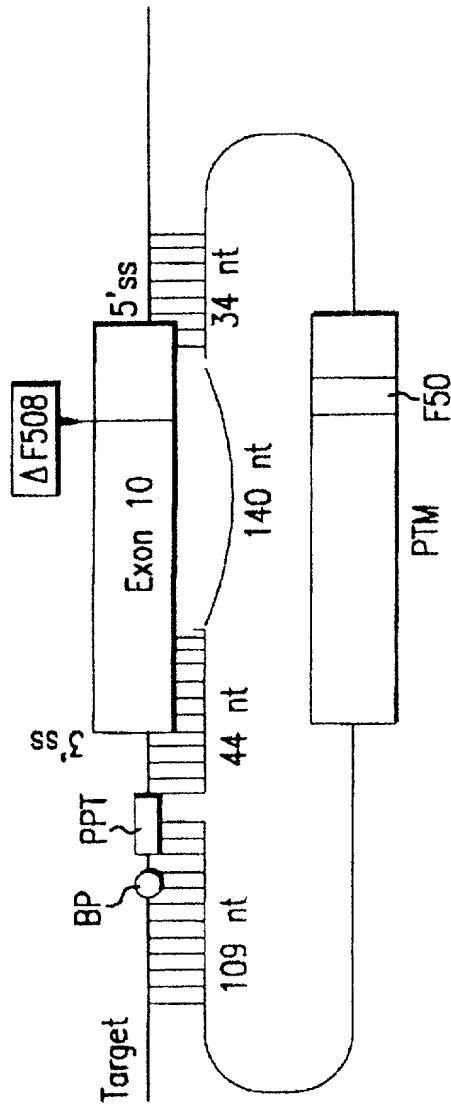


FIG.30

42 89

PTM with a long binding domain masking
two splice sites and part of exon 10
in a mini-gene target



ACGAGCTTGCTCATGATGATCATGGCCGAGTTAGAACCAAGTGAAGCAAGATCAAACATTCCCG
GGCCCATCAGGCTTTCAGCCAATTCAGTGGATCATGCCCGTACCATCAAGCAGAACATAAT
CTTCGGCGTCAGTTACGAGTACCGAGTACCGCTATCCCTGGCTGATTAAGGCCTGICAGTGGAGCAG
MCU in exon 10 of PTM
88 OF 192 (46%) bases in PTM exon 10 are not complementary to
its binding domain (bold and underlined).

FIG. 31

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Sequence of a double
Trans-spliced product

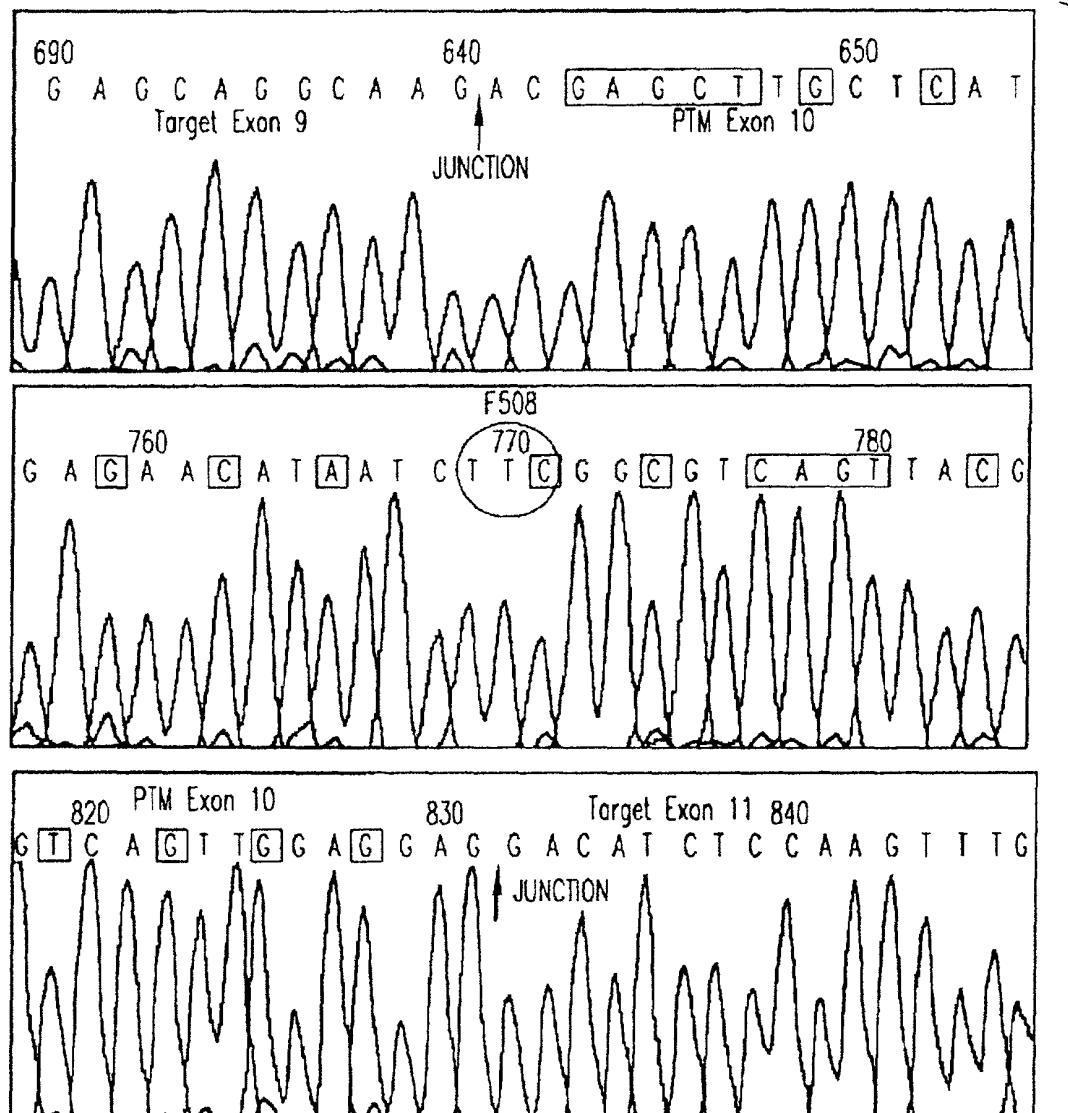


FIG.32

EE
of 91

CF-TR Repair: 5' Exon-Replacement schematic diagram of a PTM binding to the splices site of intron 10 of a mini-gene target

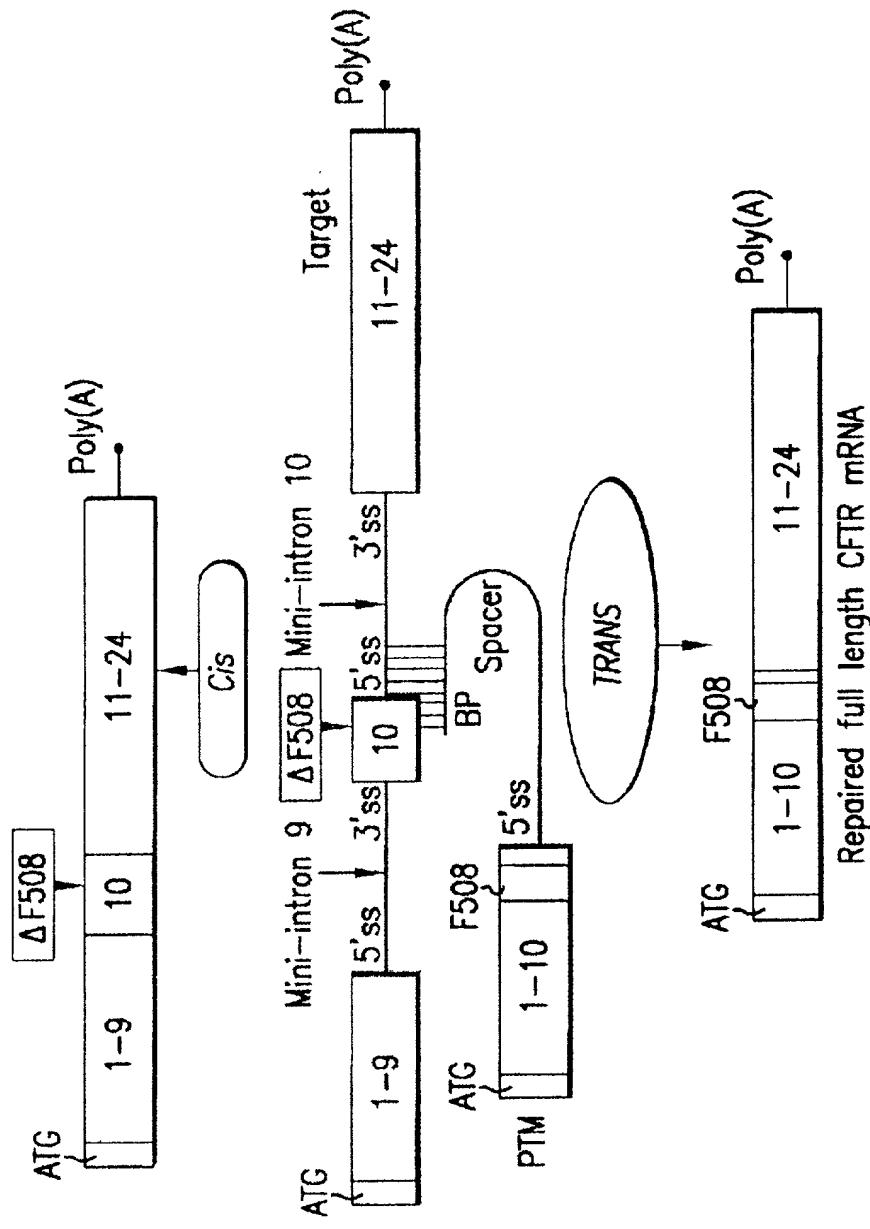


FIG. 33

PTM with a short binding domain masking a single splice site in a mini-gene target.

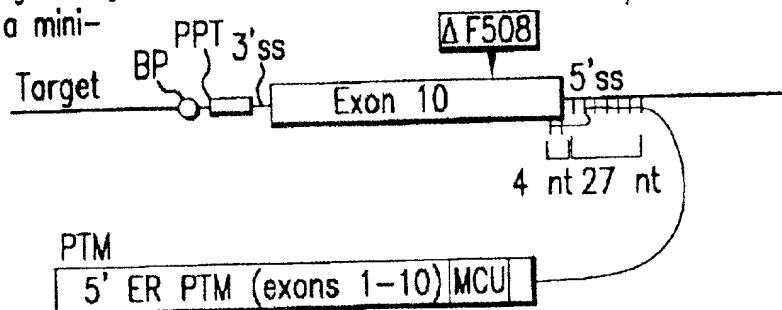


FIG.34A

PTM with a long binding domain masking two splice sites in a mini-gene target.

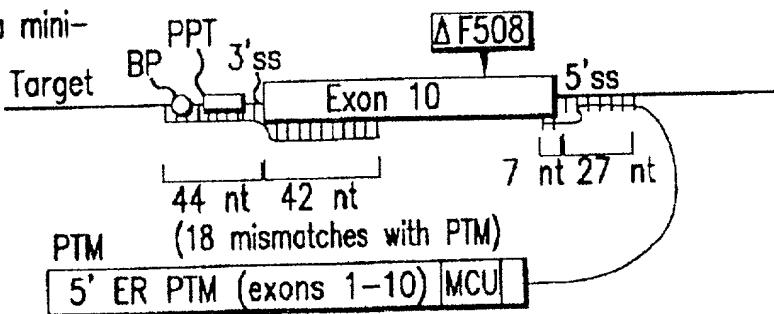


FIG.34B

PTM with a long binding domain masking two splice sites and the whole of exon 10 in a mini-gene target.

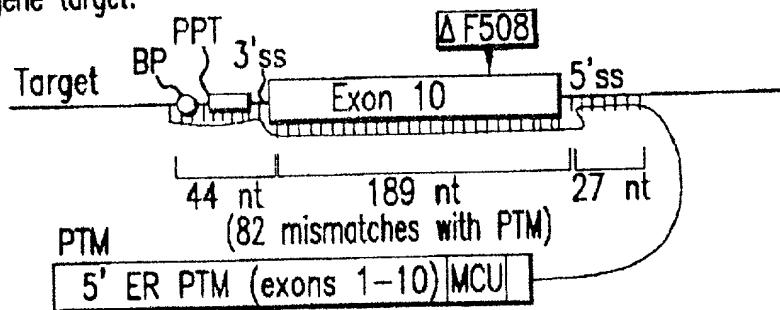
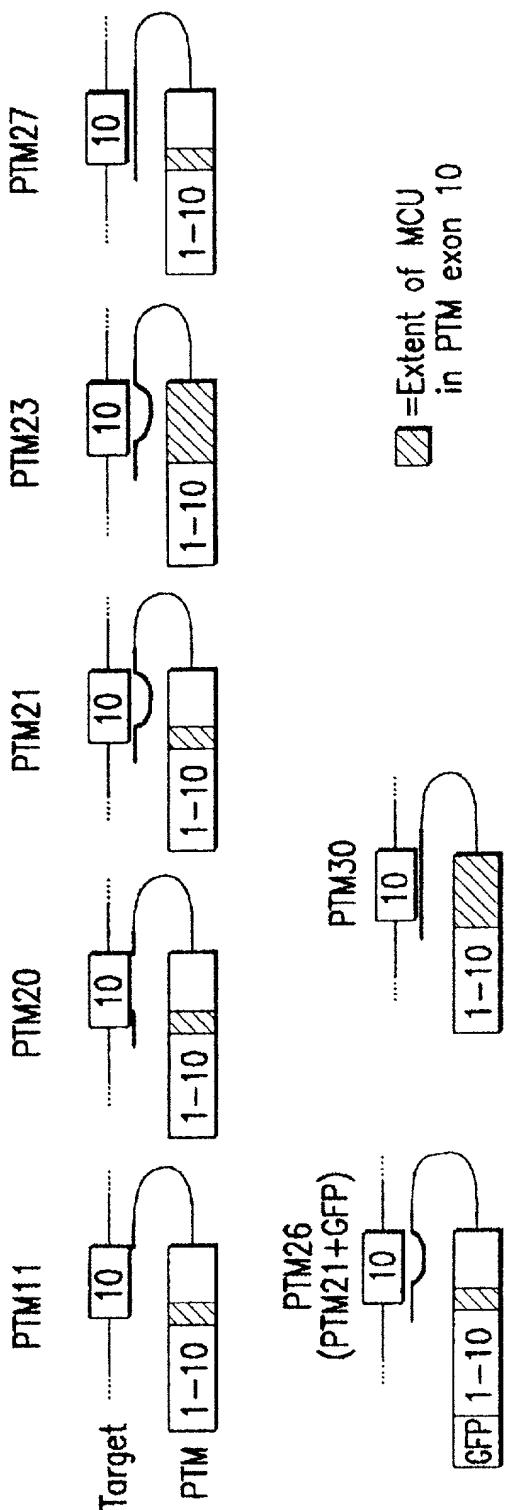


FIG.34C



MCU in exon 10 of PTM
88 of 192 (46%) bases in PTM exon 10 are not complementary to its binding domain.

ACGAGCTTGCTCATGATCATGGCGAGT~~TAGAACCAAGT~~GAAGCAAGATCAAACATTCCC
GCCGCATCAGCTTTGCAGCCAA~~T~~TCAGTTGATCATGCCGGIACCATCAAGGAGAACATAAT
CTTGGCGTCAGCTACGACTACGGCAAGTACCCGTATCCCTGGTATTAGGCCCTCTCAGTTGGAGGAC

FIG. 35

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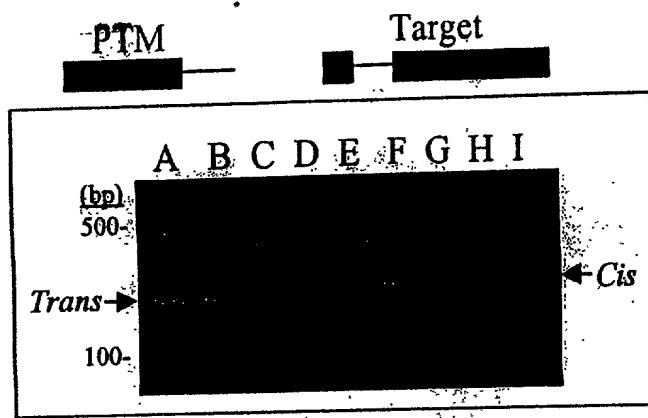


FIG. 36 A

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Cis-spliced product
[Primers CF1+CF111]

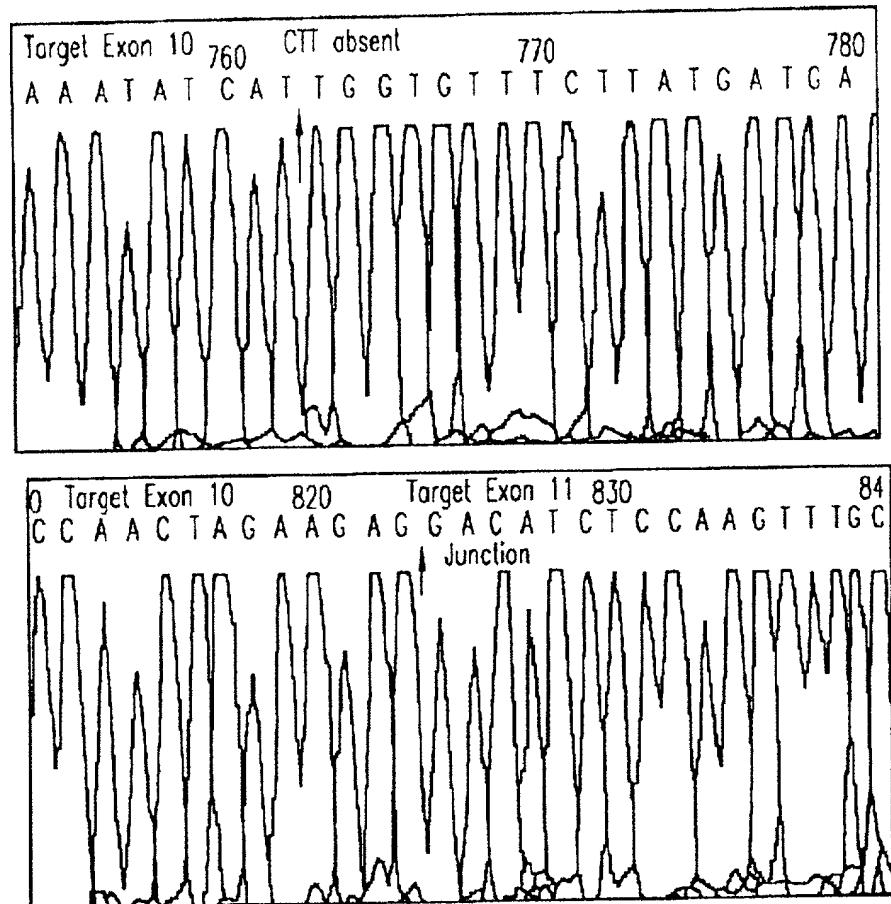


FIG.36A-1

Trans-spliced product
 [Primers CF93+CF111]

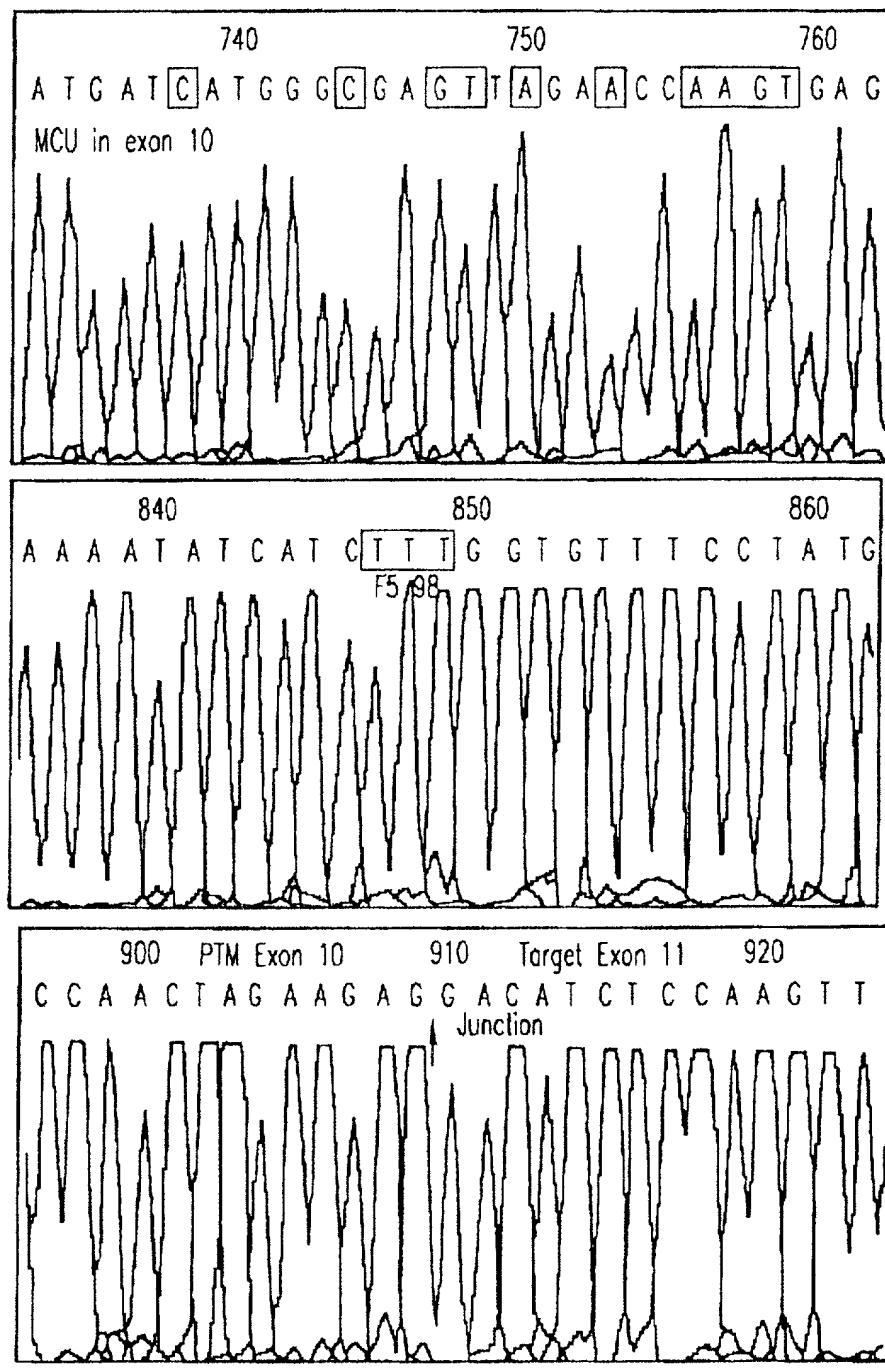


FIG.36B

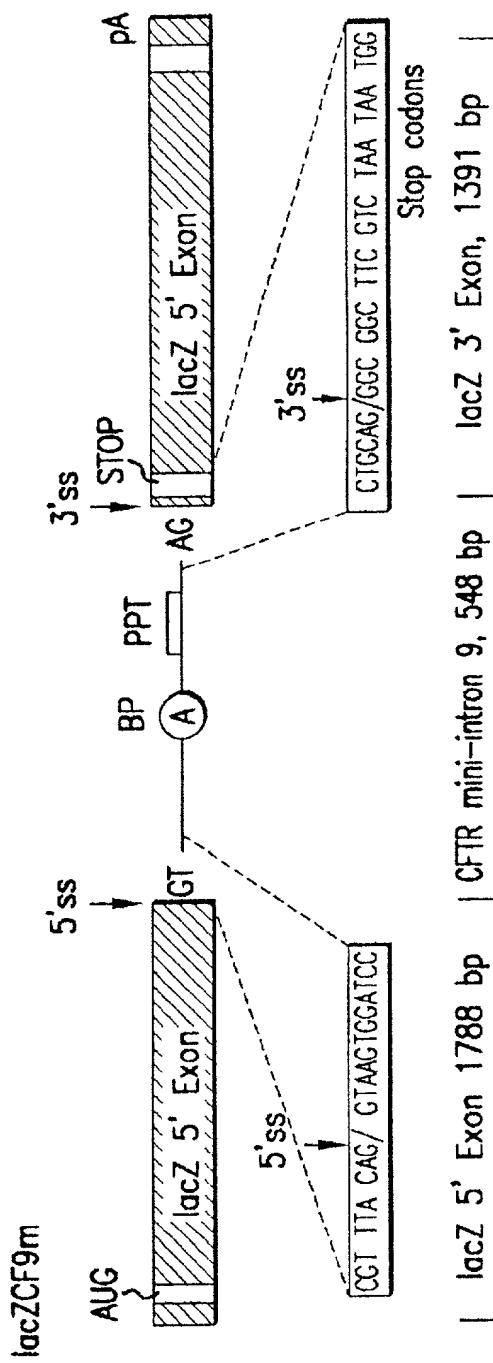


FIG.37A

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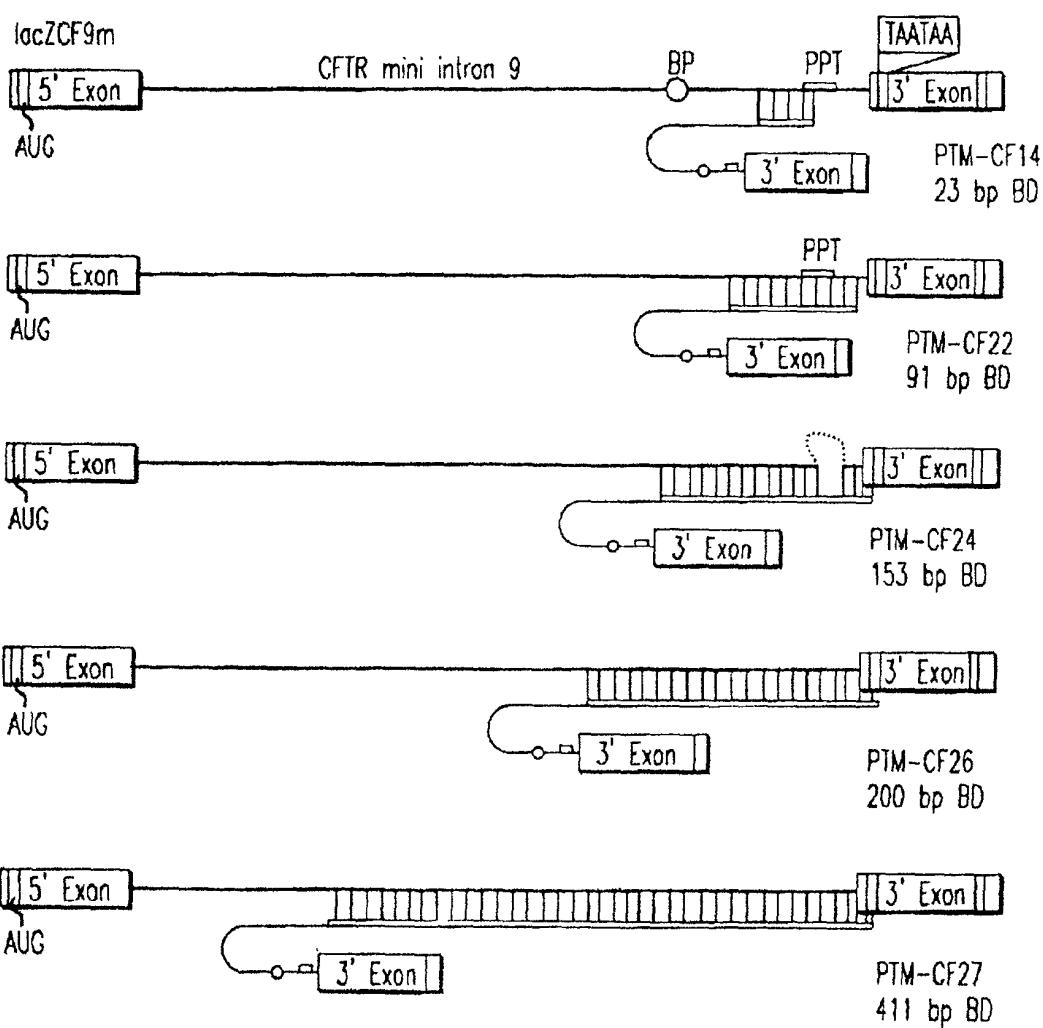
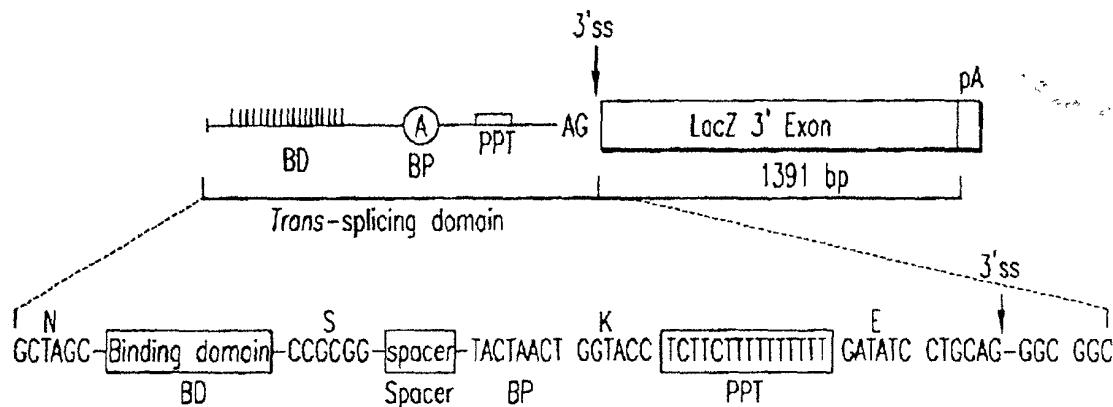


FIG.37B

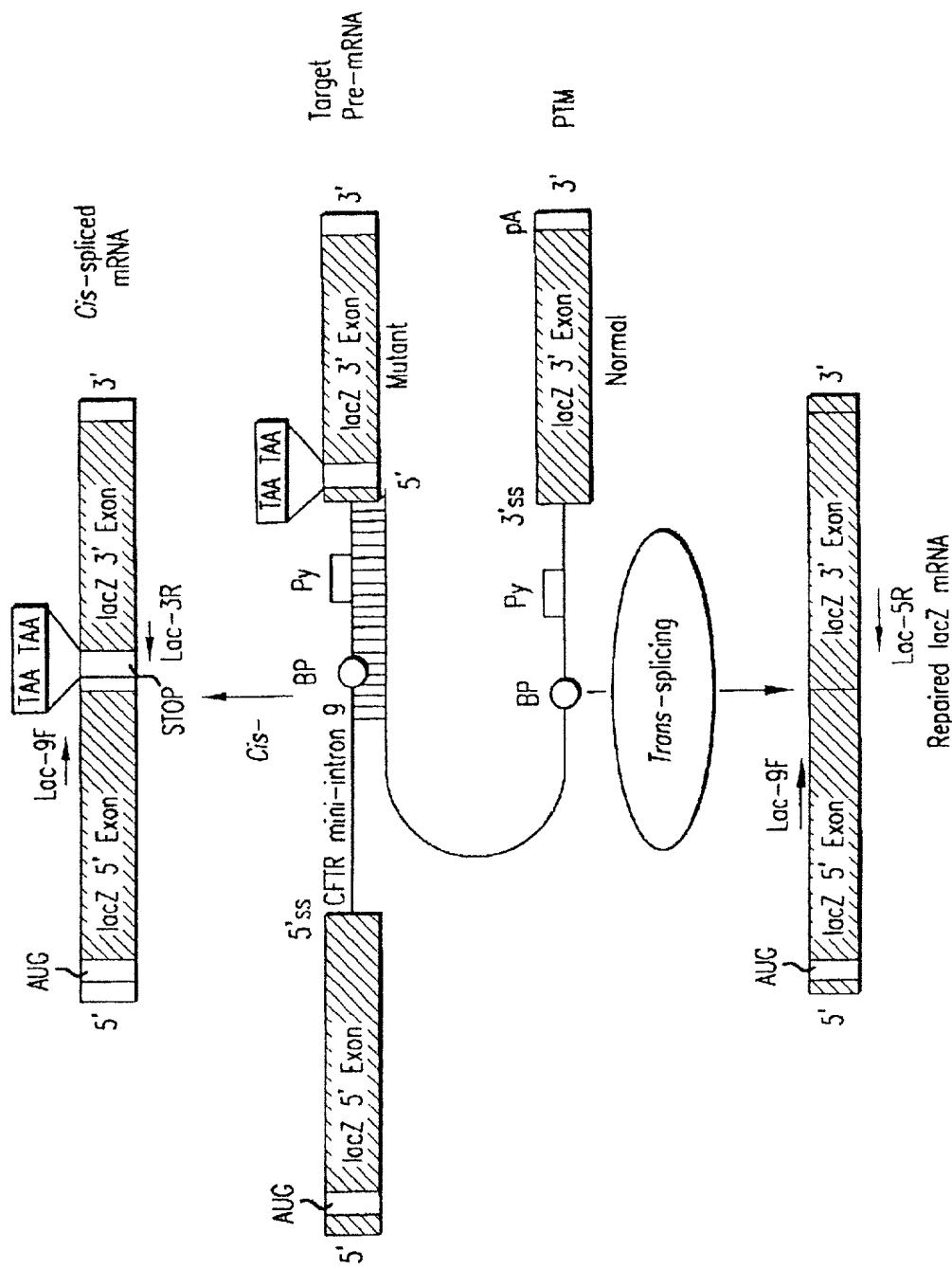


FIG. 37C

A

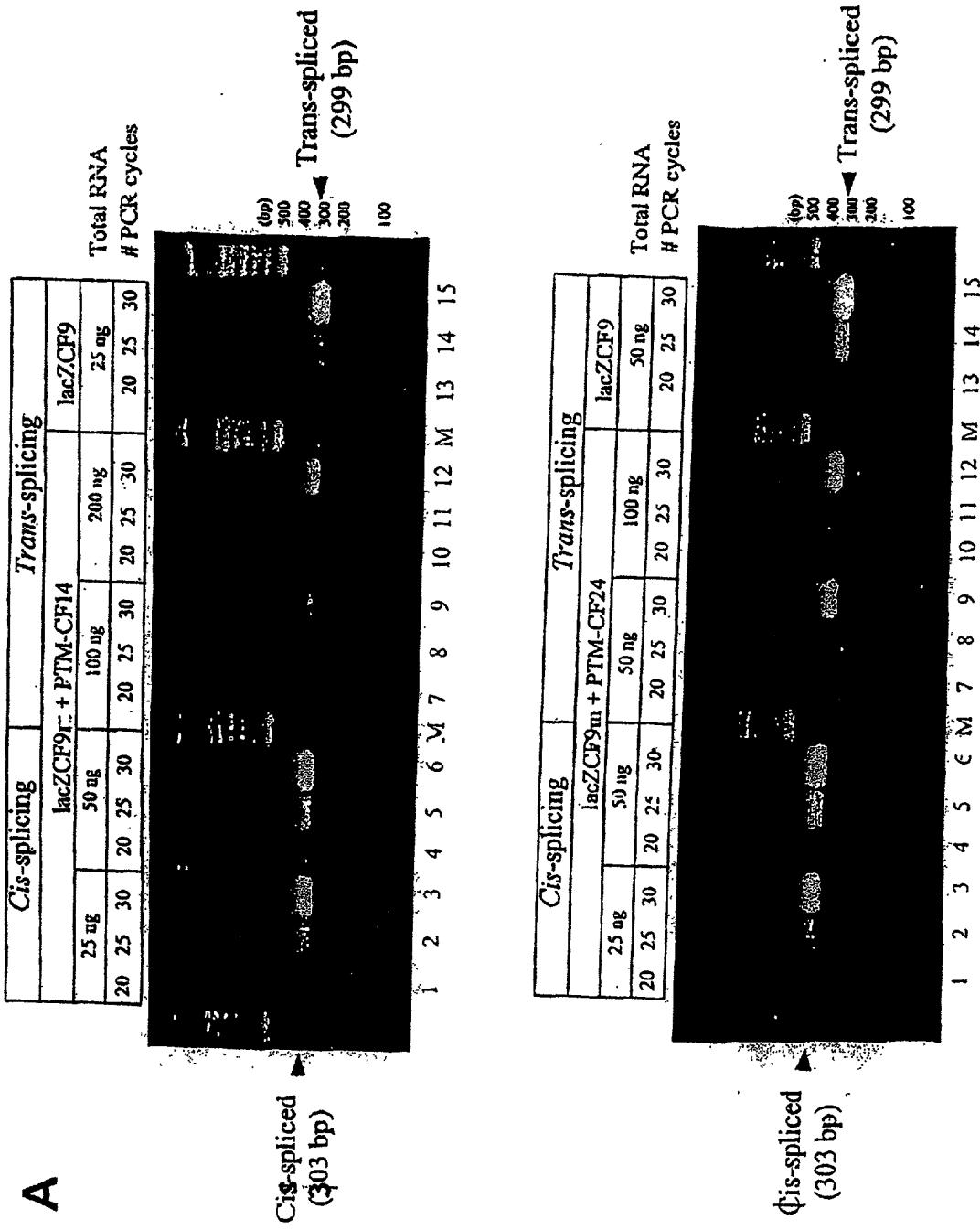


Fig. 38A

54 ♀ 91

B

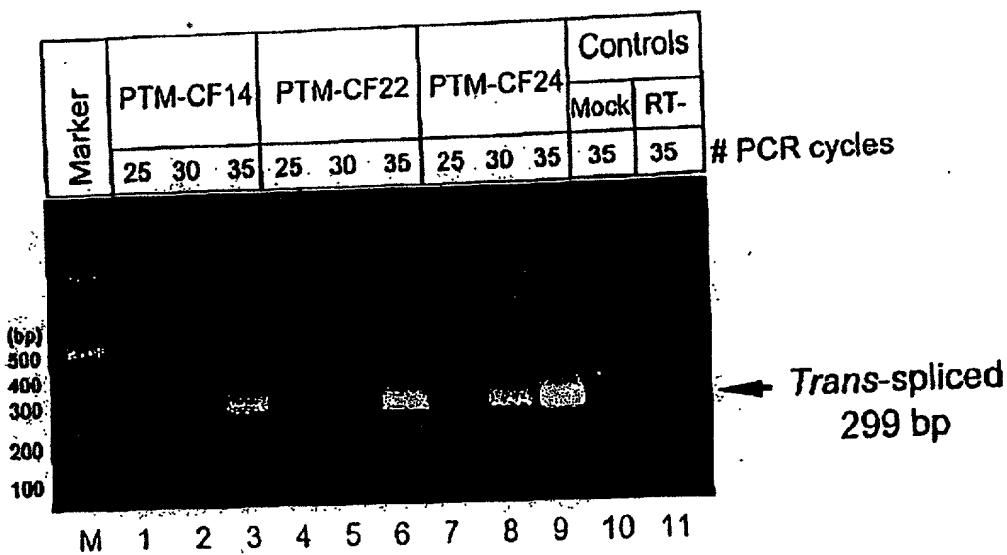


FIG. 38B

55 β 91

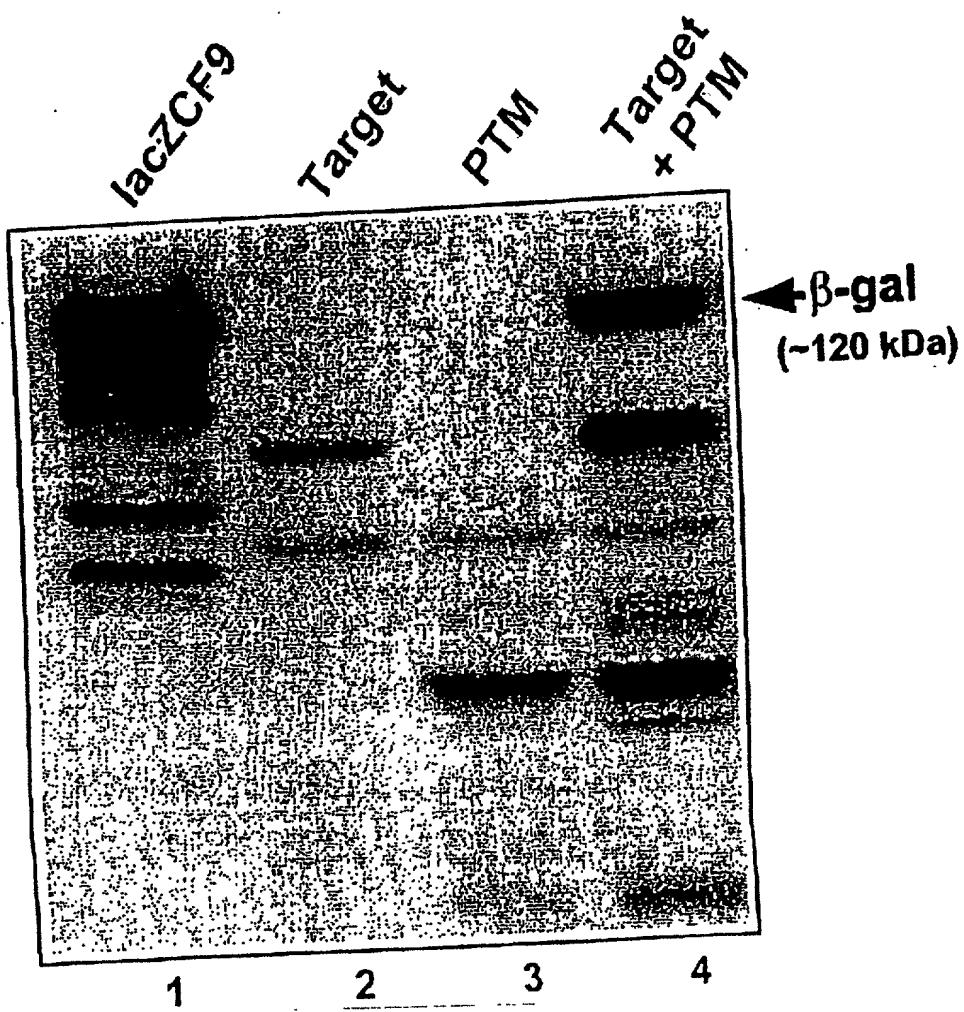


FIG. 39

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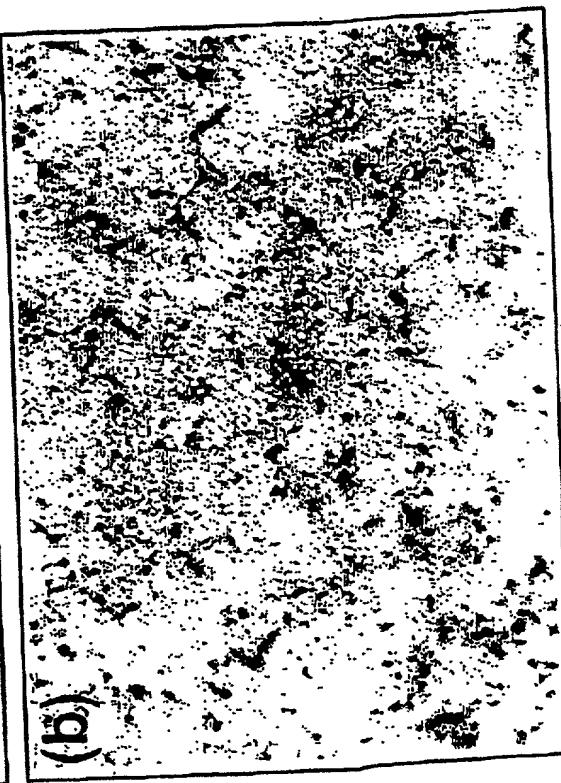
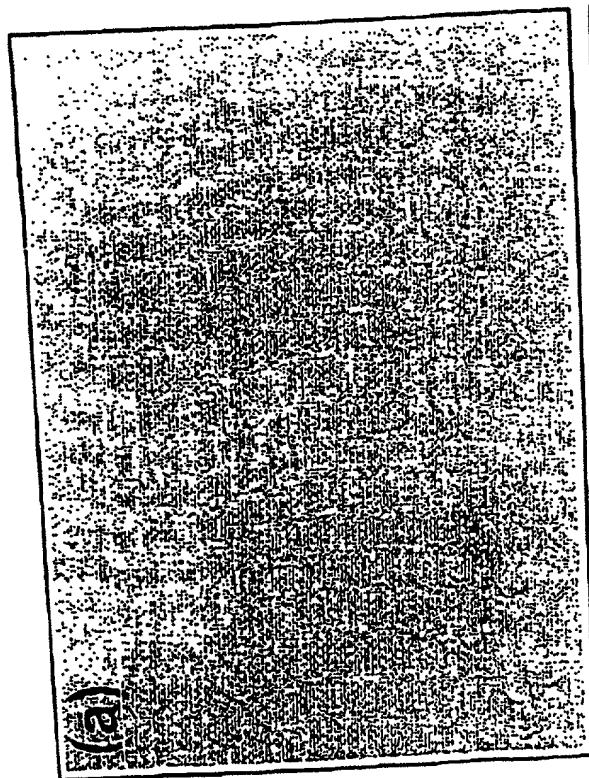


FIG. 40A

A

57 of 91

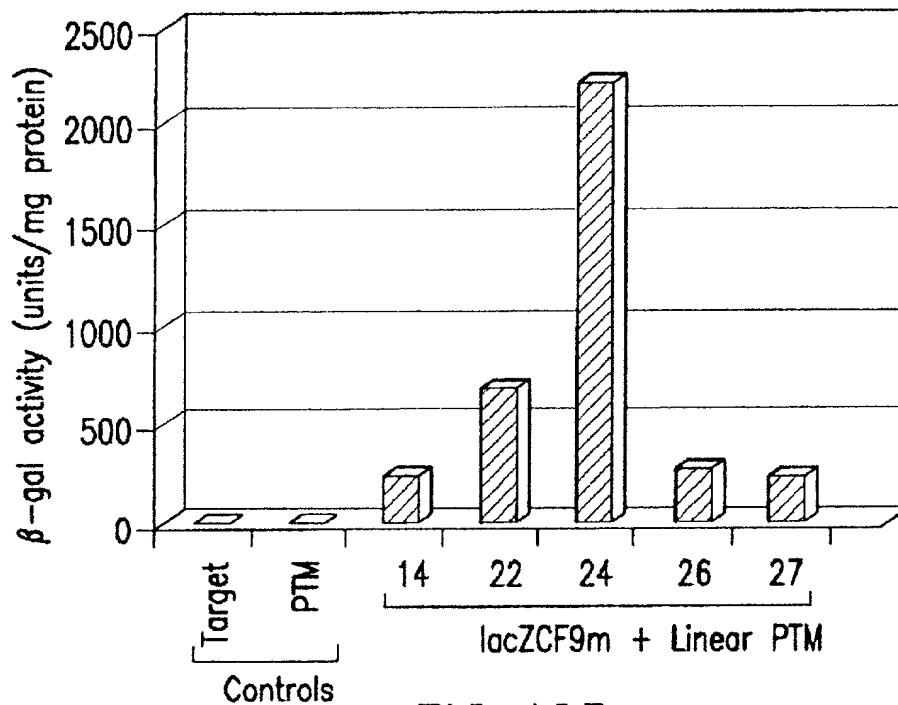


FIG.40B

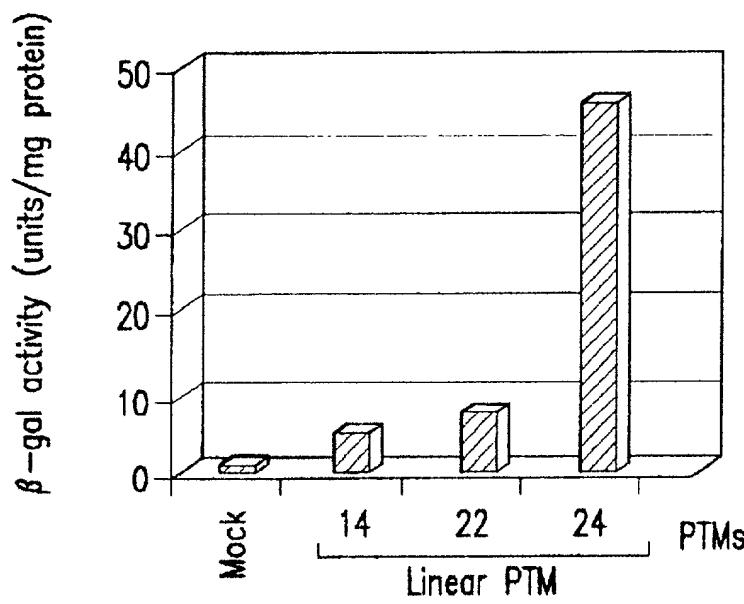


FIG.40C

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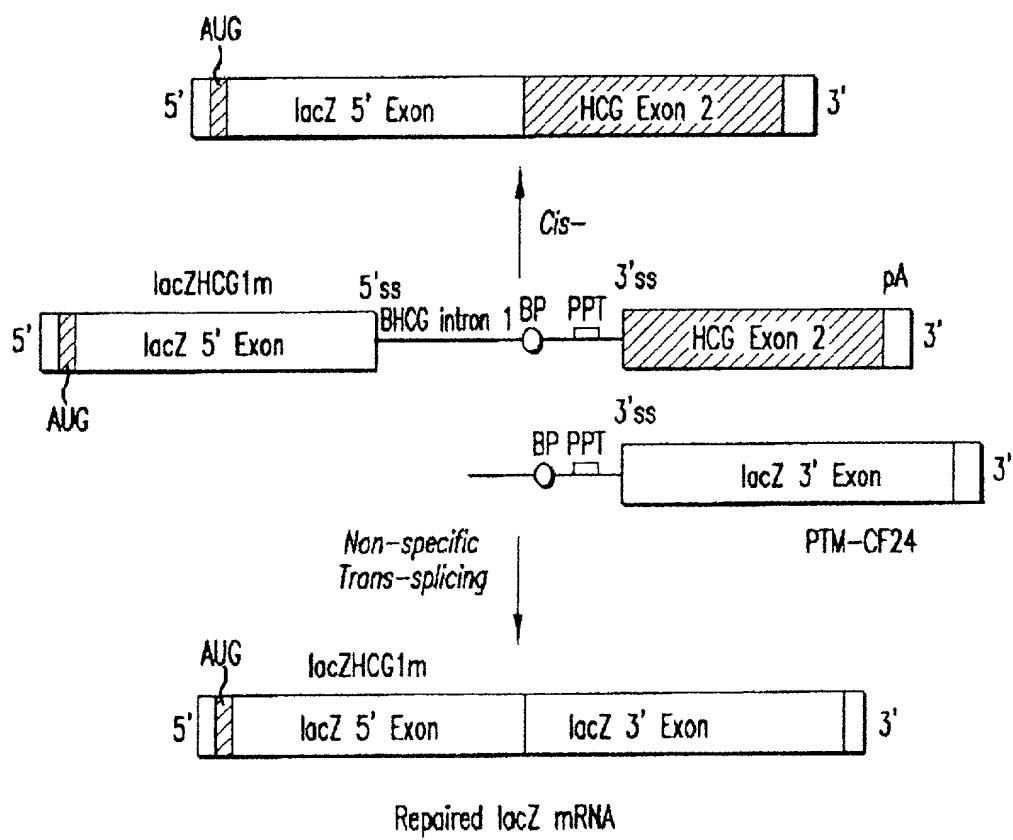
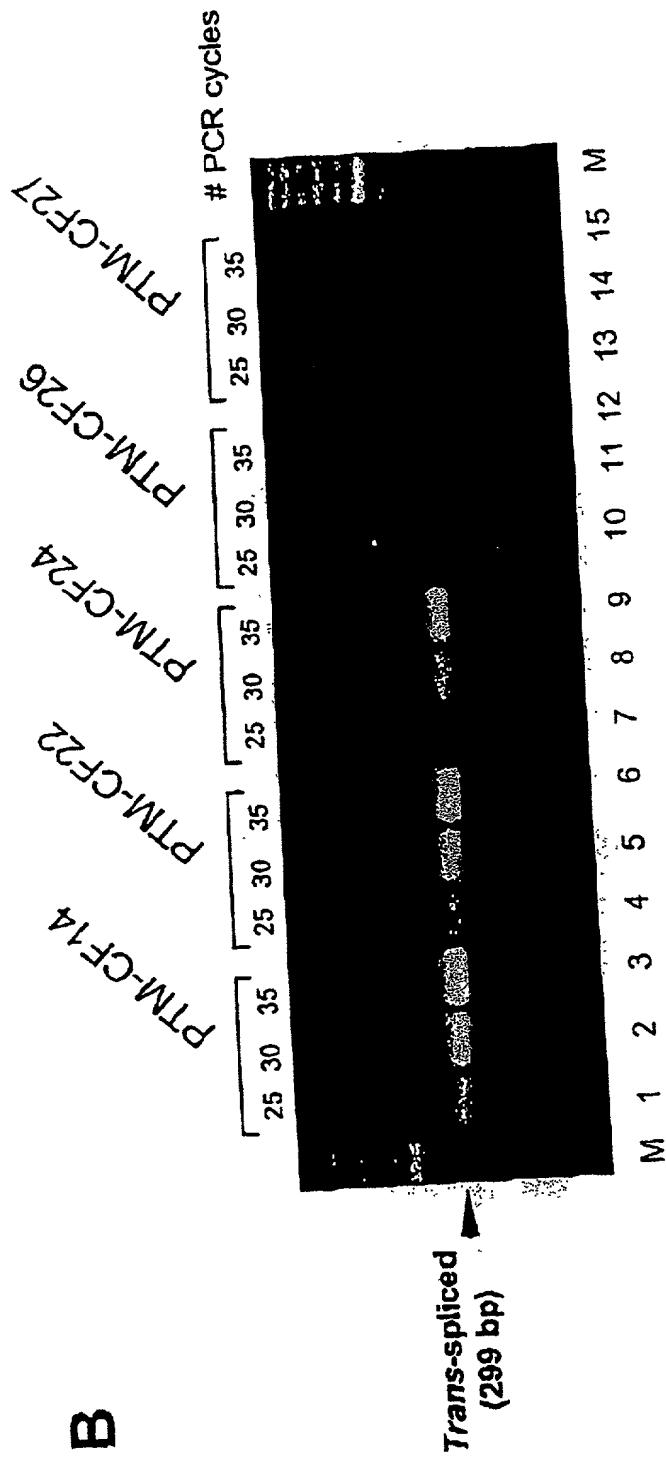


FIG.41A

Figure 4rB



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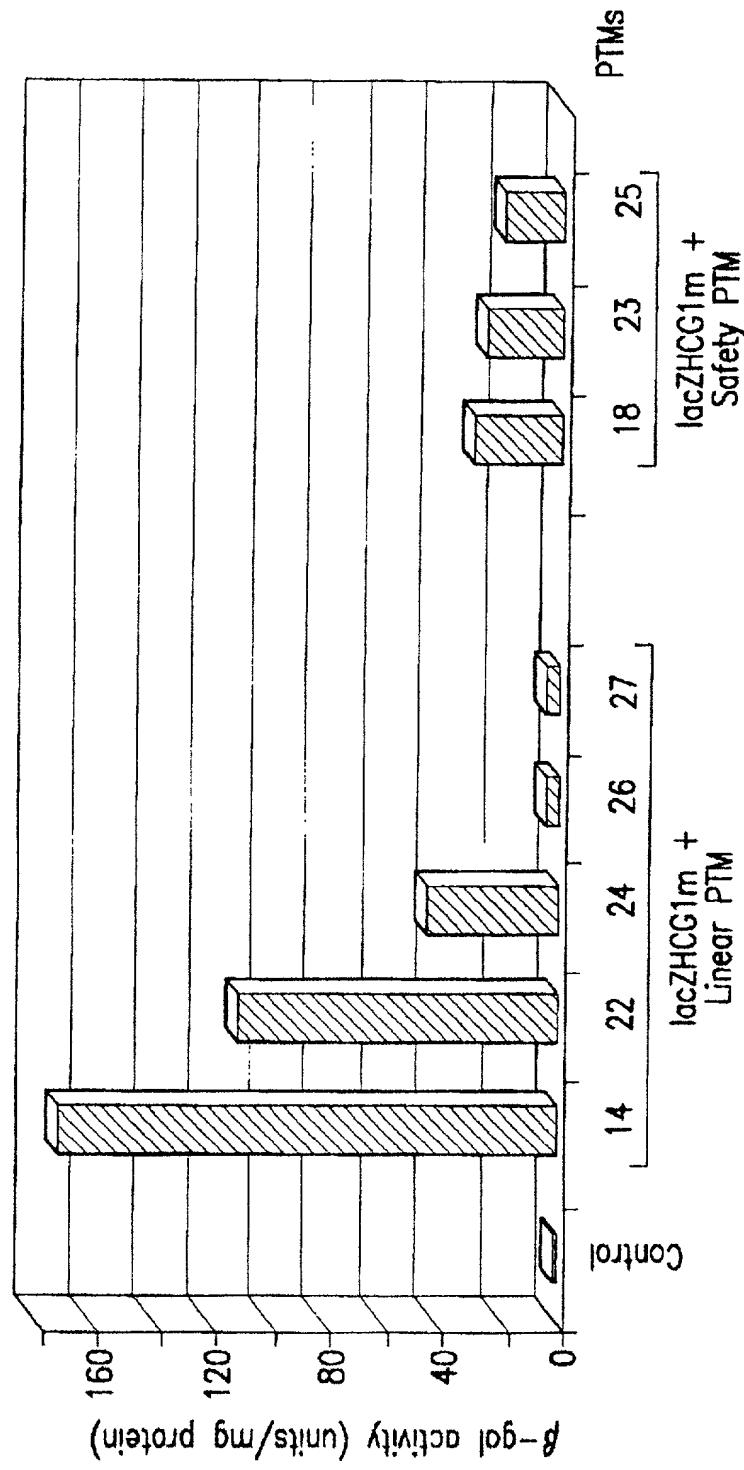


FIG. 41C

Exons

1-10

ATGCAGAGCTGGCTCTGAAAAGCCAGCGTGTCTCAAACTTTTCAAGCTGGACCAGACCAATTGAGGAAG
GATACAGACAGGCCCTGGAATTGTCAGACATAACCAAAATCCCTCTGTTGATTCTGCTGACAATCTATCTGAAAATT
GGAAAGAGAATGGGATAGAGAGCTGGCTCAAAGAAAAATCCTAAACTCATTAATGCCCTCGGGATGTTTCTGG
ACATTATGTTCTATGAAATCTTTATATTAGGGAAGTCACCAAAGCACTACAGCCTCTTACTGGAAAGAATCA
TAGCTCCTATGCCCGATAACAAGGAGGAACGCTCATCGGATTATCTAGGCATAGGCTTATGCCCTCTTTAT
TGTGAGGACACTGCTCTACACCCAGCCATTGGCCTCATCACATTGAATGCAAGTGAGAATAGCTATGTTAGT
TTGATTATAAGAAGACTTAAAGCTGTCAGCGTGTCTAGATAAAATAAGTATTGACAACCTGTTAGTCTCCTT
CCAACAACCTGAACAAATTGATCAAGGACTTCGATTGCCACATTCTGTTGGAATGCCACTCTTCAACTGCCACTCT
CATGGGCTAATCTGGACTGTTACAGGCGTCTGCCCTCTGTTGACTTGGTTCTGATAGTCCTGCCCTTTCA
GCTGGCTAGGGAGAATGATGAGTACAGAGATCAGAGCTGGGAAGATCAGTGAAAGACTTGTGATTACCTCAG
AAATGATCGAGAACATCCAATCTTAAGGCATACTGCTGGAAAGCAATGGAAAAATGATGAAACTTAAGACA
AACAGAACTGAAACTGACTCGGAAGGCAGCCATGTGAGACTTCAATAGCTCAGCCTCTCTCAGGGTTCTT
GTGGTTTTATCTGCTCCATGCCACTAATCAAAGGAATCATCCTCCGAAAATTACCCACCATCTCATTCT
GCATTGTTCTGCCATGGCGTCACTGGCAATTCCCTGGCTCTACAAACATGTTGACTCTGGAGCAATAAA
CAAATACAGGATTCTTACAAACCAAGAATATAAGACATTGGAATATAACTAACGACTACAGAAGTAGTGATGGAG
AATGTAACAGCCTCTGGAGGAGGATTGGCAATTTCAGAAAGCAAAACAAACAATAACAATACAAAAACTT
CTAATGGTGATGACAGCCTCTTCAGTAATTCTCACTTCTGTTGACTCCTGCTGAAAGATATTAAATTCAAGAT
AGAAAGAGGACAGTTTGGCGTTCGACTGGACAGGCAAGAAGAGCTTGCTCATGATGATGATGCCGAG
TTAGAACCAAGTGAAGCAAGATCAAACATTCCGGCCCATCAGCTTITGCAAGCAATTCACTGGATCATGCCGTA
CCATCAAGGAGAACATAATCTTGGCGTCACTTACGACAGTACCGCTATCCCTCGTATTAGGCCTGTCAGTTGG
GGAG

Trans-splicing domain

GTAAGATATCACCGATATGCTAACCTGATTGGCCCTCGATACCTAACATCCACCGG
TCAAAACTTTCACATAATTCTTACCTCTTGAATTCACTGCTTGTGACGCCCTCTGATATCTATATTCACTATTG
GAAACACCAATGATAATTCTTAAATGGTCCCTGCCATAATCCTGGAAAATGATAACACAATGAAATTCTCCACTGT
GCTTAATTTCACCTCTGAATTCTCCATTCTCCATAATCATCATTACAACGACTCTGGAAATAAAACCATCATT
ATTAACTCATTCAAATCACGCT

FIG.42

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153 bp PTM24 Binding Domain:

Nhe I

GCTAGC-AATAATGACCAAGCCGCCCTCACGCTCAGGATTCACTTGCCCTCCAATTATCATCCTAAGCCAGAACGCTATA

TTCTTATTTGTAAGATTCTATTAACTCATTTGATTCAAAATATTAAATAACTTCCTGTTCACCTACTCTGCTATGC

Sac II

AC-CCCCGG

FIG.43A

Trans-splicing domain

AATAATCACGAAAGCCCCCCCACCCCTCAGGATTCACTGCCCTCAATTATCATCTAACGAGAAGTGTATATTCTTA
TTTGAAAGATCTATTAACTCATTCATTCAAATATTAAAATACTTCCCTTACCTACTCTGCTATGCACCCCG
GGAACATTATTATAACGTTGCTCGAATACAACTGGTACCTTCTTTTTTGATATCCTGCAG

Exons 10-24

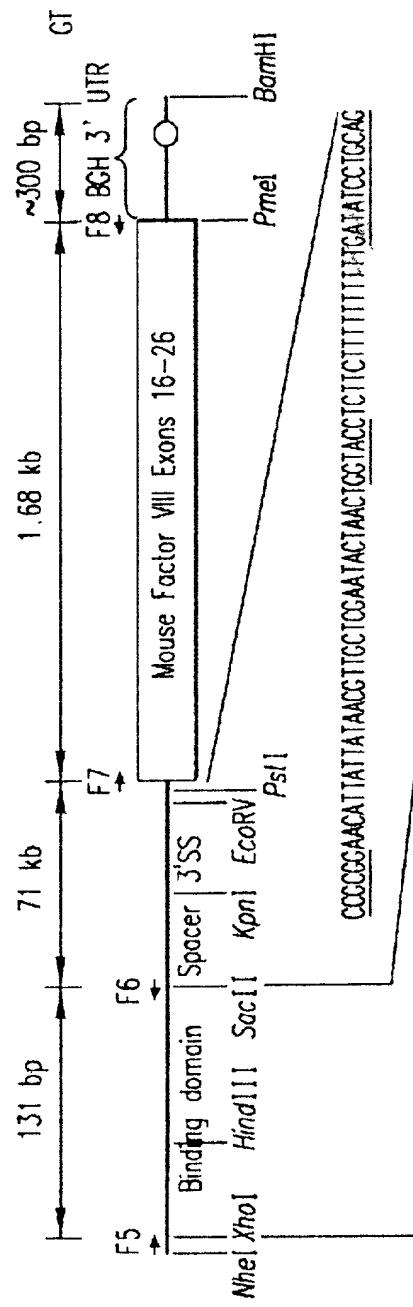
ACTTCACCTCTAAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTAAACCACAGTCGAAGAATTCTCATCT
 GTTCTCACTTTCCCTGGATTATGCCCTGGCACCATTAAAGAAAATATCATCTTGGTCTTCCTATGATGAATATAGATA
 CACAAGCGTCATCAAACATGCCAAGTAAAGAGGACATCTCAAGTTGAGAGAAAAGACAATATACTTCCGACAA
 GGTGGAATCACACTGACTGGAGGTCAACGAGCAAGAATTCTTAGCAAGAGGACTATAACAAAGATGCTGATTGTATT
 TATTAGACTCTCTTTGGATACCTAGATTTAACAGAAAAAAATATTGAAAGCTGTCTAAACTGATGCC
 TAACAAAATAGGATTTGGTCACTTCTAAATGGAACATTAAAGAAACCTGACAAAATATTAAATTTCATGAAGGT
 ACCACCTATTTTATGGGACATTTCAGAACTCCAAAATCTACACCCAGACTTAGCTCAAACACTCATGGGATGTTGATT
 CTTCCGACCAATTAGTCAGAAAGAAATTCAACTCAACTGAGACCTACACCGTTCTCATTAGAAGGAGATGC
 TCCTGTCTCCCTGGACAGAAACAAAAAAACATCTTAAACAGACTGGAGACTTGGGGAAAAAGGAAGAATTCTATT
 CTCATCCAATCAACTCTATACGAAAATTTCATGTGAAAAGACTCCCTACAAATGAATGCCATCGAAGAGGATT
 CTGATGAGCCTTAGAGAGAAAGCCTGTCCTAGTACCAAGATTCTGAGCAGGGAGAGGCGATACTGCCCTCGCATCACCGT
 GATCAGCACTGCCCTCACGTCAGGAGGAGCTGTCCTGACACACTCACTAACCAAGGT
 CAGAACATTACCCAAAGACAACAGCATCCACACGAAAAGTCTACTGCCCTCAGGCAAACCTGACTGAACCTGATA
 TATATTCAAGAAGTTATCTAAGAAACTGGCTGGAAATAAGTGAAGAAATTAAACGAAGAAGACTTAAAGGAGTGCTT
 TTTGATGATATGGAGAGCATACCAAGCACTGACTACATGGAACACATACCTTCGATATATTACTGTCACAGAGCTTA
 ATTTCGCTAATTGGTCTTACTAATTCTGGAGGTTGCTTGTGCTTGGTTGCTGTCGGCTCTGGAA
 ACACCTCTCTCAAGAACAAAGGAATAGTACTCATAGTAGAAATAACAGCTATGCACTGATTACCCAGCACCAGTTC
 GTATTATGTGTTTACATTACGTTGGGAGTAGCGGACACTTGTGCTGCTATGGGATTCTTCAGAGCTTACACTGGT
 CATACTCTAATCACACTGTCGAAAATTTCACCCACAAATGTTACATTCTGTTCTCAACCCATGTCAACCCCTCA
 ACACGTTGAAAGCAGTGGCATTCTTAATAGATTCTCAAAGATAAGCAATTGGATGACCTTCTGCCCTTACCAT
 ATTGACTTCATCCAGTTGTTATTAAATTGTGATTGGAGCTATAGCACTTGTGCACTTTACAACCCCTACATCTTGT
 GCAACAGTGCCAGTGTAGTGGCTTTATTATGTTGAGGCAATTCTCAAACCTCACAGCAACTCAAACAACTGG
 AATCTGAAGGAGGAGTCCAATTTCACTCATCTGTTACAAGCTTAAAGGACTATGGACACTTGTGCCCTGGAGC
 GCAGCCTTACTTCAAACCTCTGTTCCACAAAGCTCTGAATTACATACTGCCAAGTGGCTTGTGACCTCTGTCACACTG
 CGCTGGTTCCAAATGAGATAAGAAATGTTGCTCATCTTCTCATGCTGTTACCTCTGTCATTCCATTAAACACAG
 GAGAACGGAGAACGAAAGACTTGTATTCTCACTTACCCATGAATATCATGACTACATTGCACTGGGCTGAAACTC
 CAGCATAGATGTGAGTGTGATGCCATCTGAGCCACTCTTAAAGTCTATTGAGATGCACTGCAACAGAACGTAACCT
 ACCAACTCAACCAACCATACAAGAATGCCAAGTCTGAAAGTTGATTATGAGAAATTACACAGTGAAGAACGAT
 ACATCTGCCCTCAGGGGGCCAATGACTGTCAGGAACTCTGAAAGTATGCACTGCAACAGAAAATACAGAACGTTGAAATGCCATTAGA
 GAACATTCTCTCAATAACTCTGGCAGACGGTGGGCTTGGAAAGAACTGGATCAGGAAAGACTTGTGTT
 TCAGCTTTTGAGACTACTGAAACACTGAAAGGAAATCCAGATGCACTGGTGTCTGGGATTCATAACTTTGCAAC
 ACTGGAGCAAACCTTGGAGTGTACACAGAAACTTATTCTGGAACTTGTGAACTTGGGATCCCTA
 TGAAACAGTGGAGTGTCAAGAAAATGGAAAGTTGCACTGAGGTTGGGCTCAGATCTGTGATAGAACACTTCTGG
 AACCTTGACTTGTCTTGGAATGGGCTGTCCTAAGCCATGCCACACAGTTGATGTCCTGGCTAGATCTG
 TTCTGACTAAGGGCAAGATCTGCTGCTTGTCAACCCAGTGCTCATTTGGATCCAGTAACATACCAAAATTAGAAC
 AACTCTAAAACAAGCATTCGCTGATTGCACTAATTCTGTGAAACACAGGATAGAAGCAATGCTGGATGCCAACAA
 TTTTGTCATAGAAGACAACAAAGTGCAGGCACTACGATTCCATCCACAAACTGCTGAAACGAGGAGGACCTCTCCGGC
 AACCCATCAGCCCCCTCCGACAGGGTGAAGCTTTCCCCACCGGAACCTCAACCAAGTCAAGCTAAGCCCCACATTGC

Histidine tag Stop

TGCTCTGAAAGAGGAGACAGAACAGAGGTCCAAGATACAAGCTTACATCATCATCATCATTAG

FIG.43B

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Binding domain (complementary to intron 15
and exon 16 nucleotides -142 to -29, -6 to +4
CTCGAGTTACCTGAACTTATTTTTAGAATTAATAAATCTTAAC
GTATCCCTCACTCTTGTATCTTATCTTATCTTATCTTATCTT

FIG. 44A

65 89 91

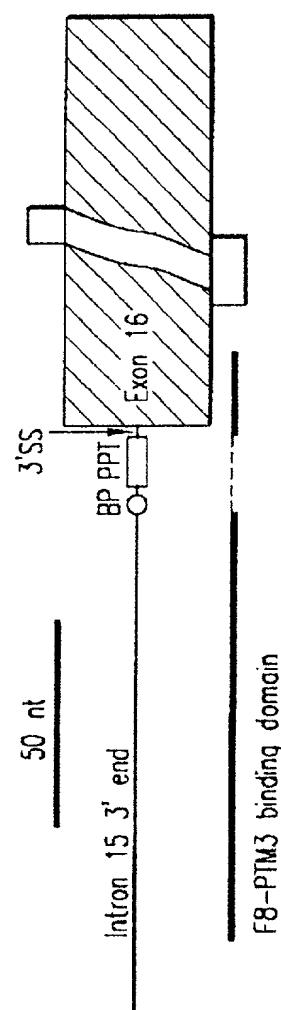


FIG. 44B

The diagram illustrates the construct's organization. It features a dashed box labeled "Extent of promoter in above construct". Inside this box is another dashed box labeled "Extent of promoter in original construct". Below these boxes is a solid box labeled "CMV enhancer". To the right of the boxes is a hatched rectangle representing "Exon 1 Intron 1 (partial)". The total length of the construct is indicated as 525 bp, with 277 bp for the promoter and 94 bp for the exon/intron region.

Extent of promoter in above construct	Extent of promoter in original construct	CMV enhancer	Exon 1 Intron 1 (partial)
525	277	94	117

$F13+F2=235+106=341$ bp
 $F13+F4=235+35=250$ bp

Chicken Beta Actin Promoter (including exon 1 and part of intron 1)

FIG. 44C

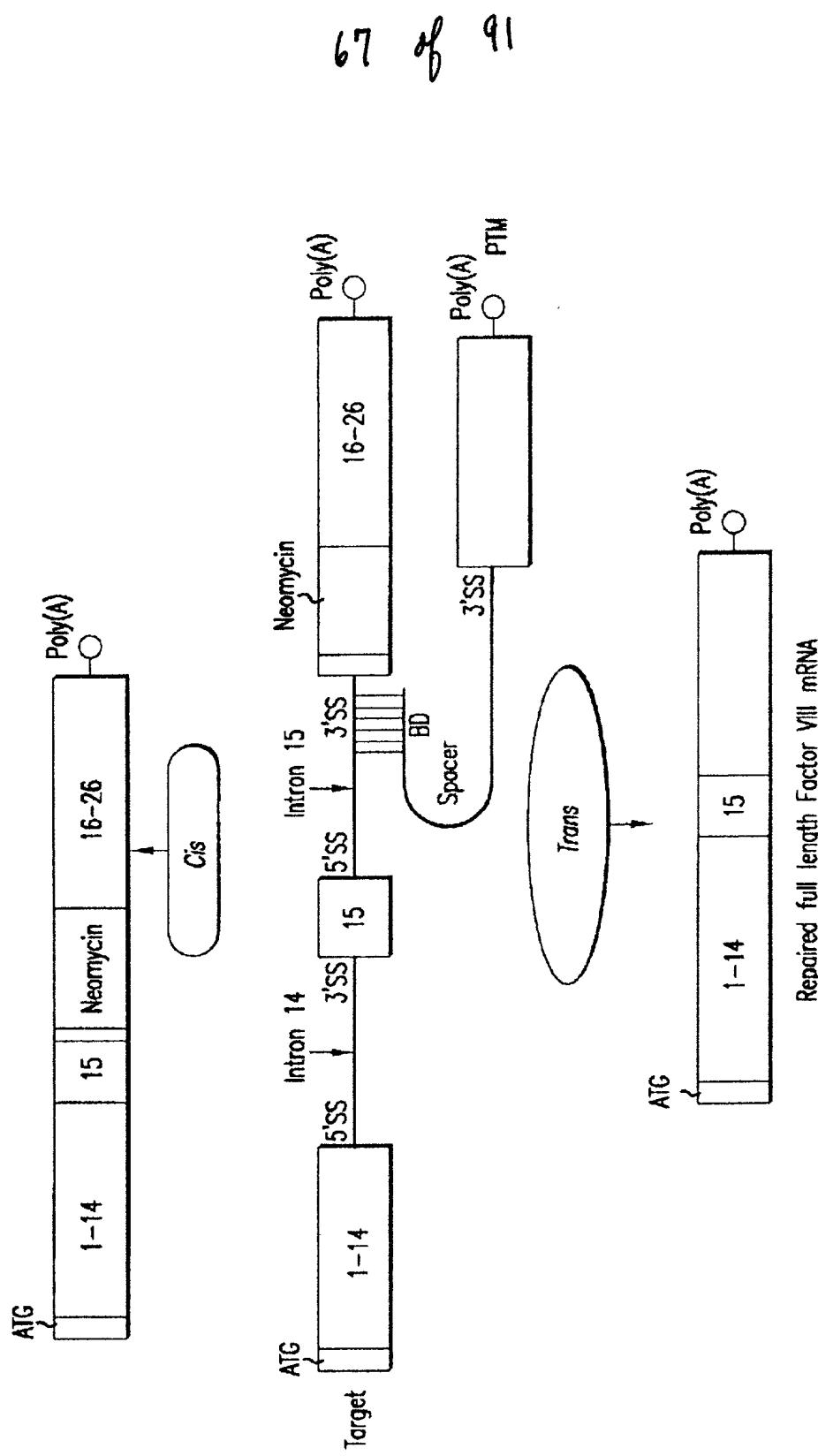
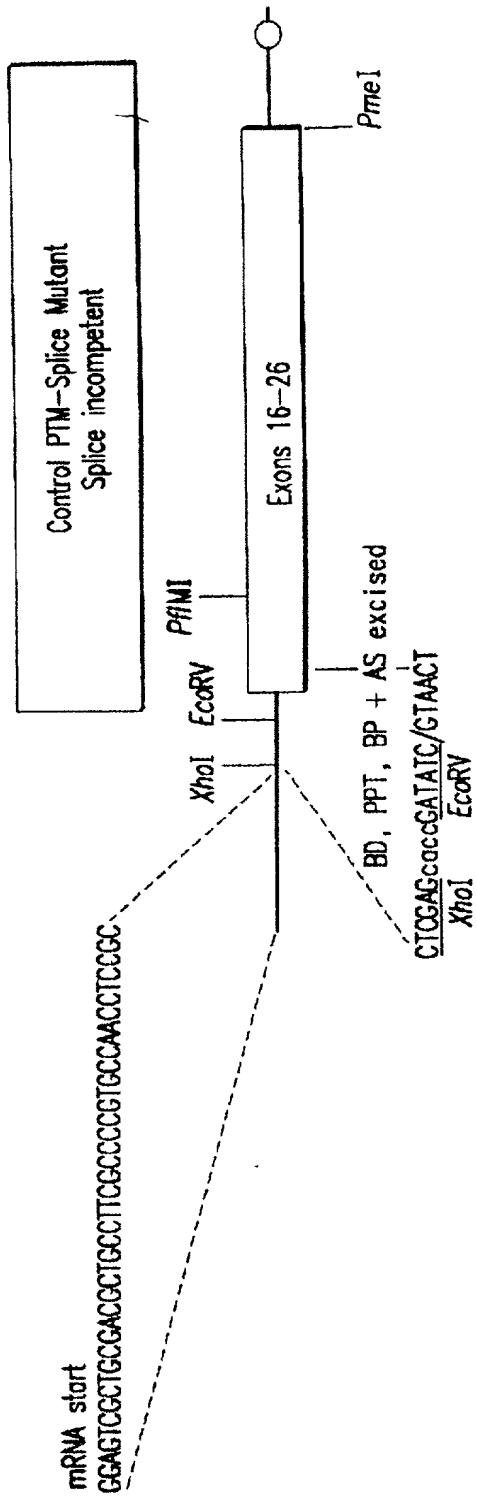


FIG. 44D



Method:

Excise TSD and part of exon 16 with XbaI and PstI and ligate in a PCR product that:

- 1) eliminates the TSD and splice acceptor site
- 2) inserts EcoRV adjacent to exon 16
- 3) restores the coding for exon 16

FIG. 45

Repair of Factor VIII
Preliminary results from one experiment

FVIII activity in Exon 16 FVII-KO mice
after IV PTM-FVII intraportal infusion
(100 μ gDNA)(n=3)

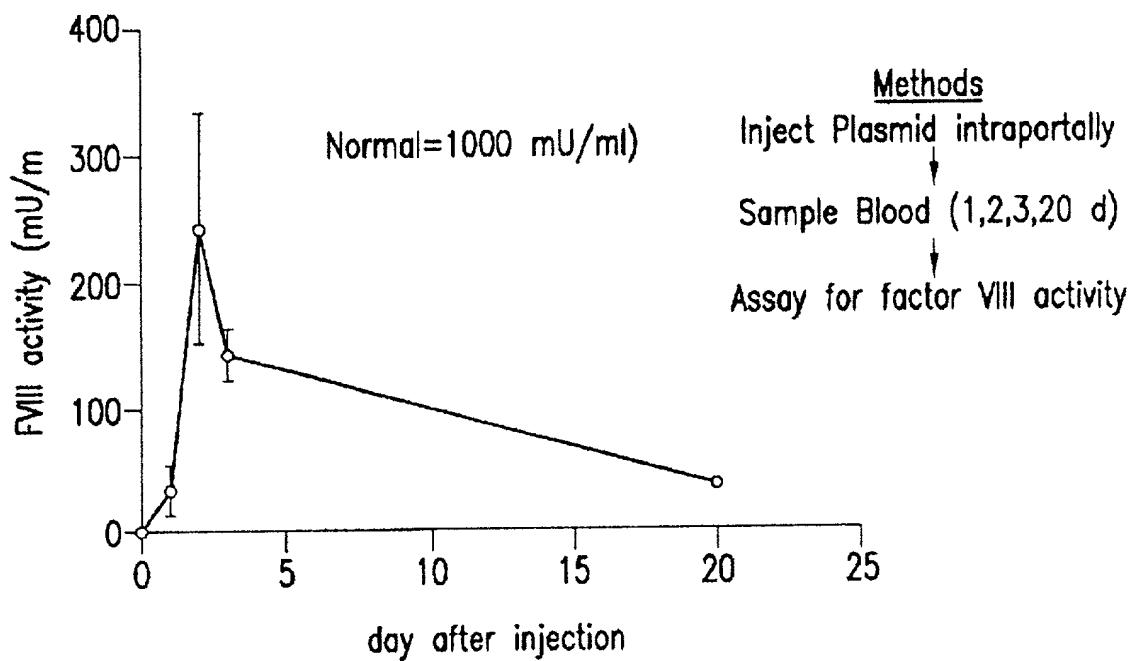


FIG.46

Detailed structure of a mouse factor VIII PTM containing normal sequences for exons 16-26 and a C-terminal FLAG tag. BGH=bovine growth hormone 3' UTR; Binding domain= 125 bp.

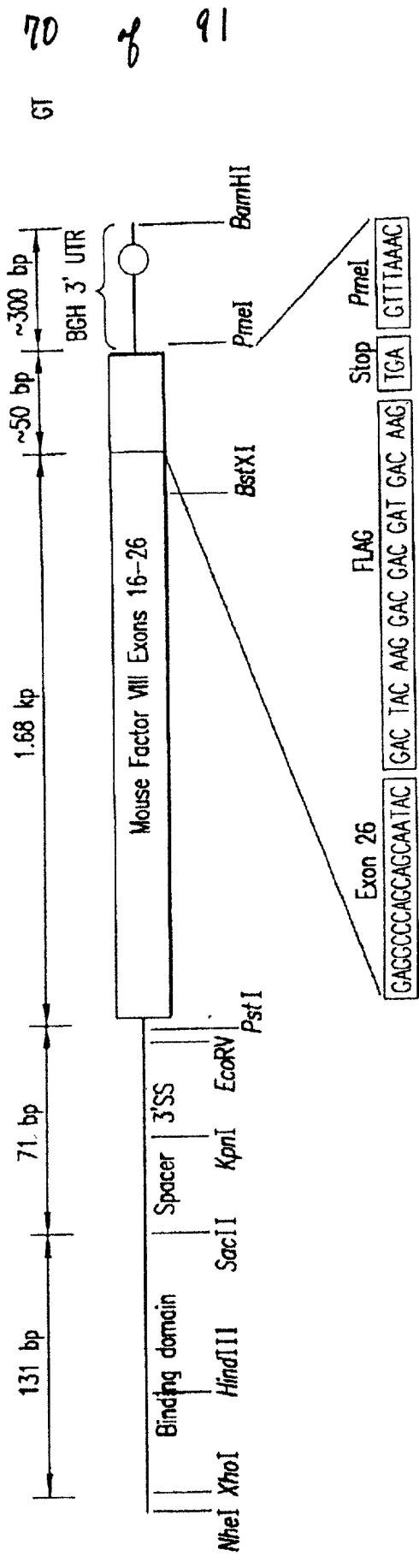
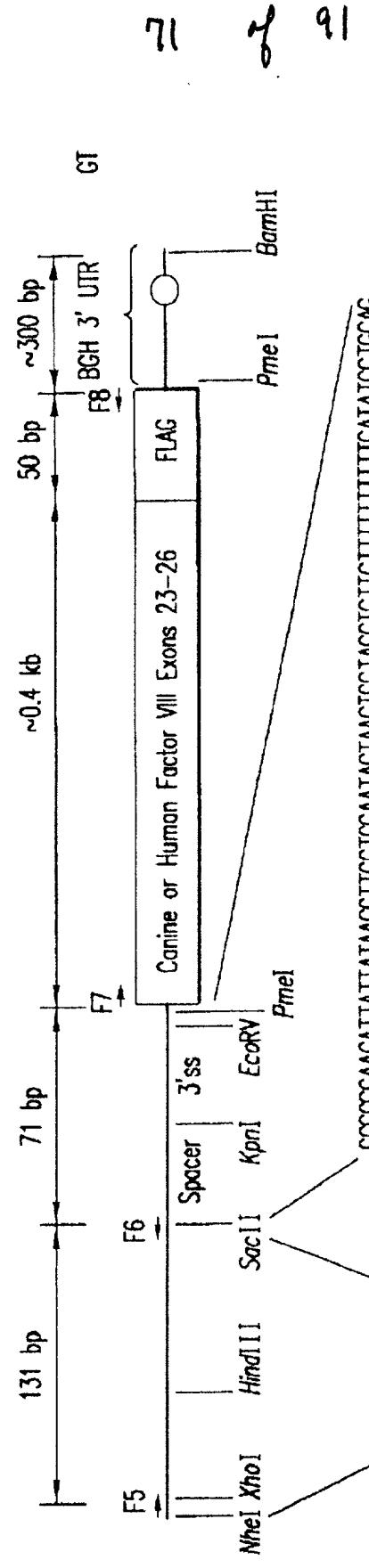
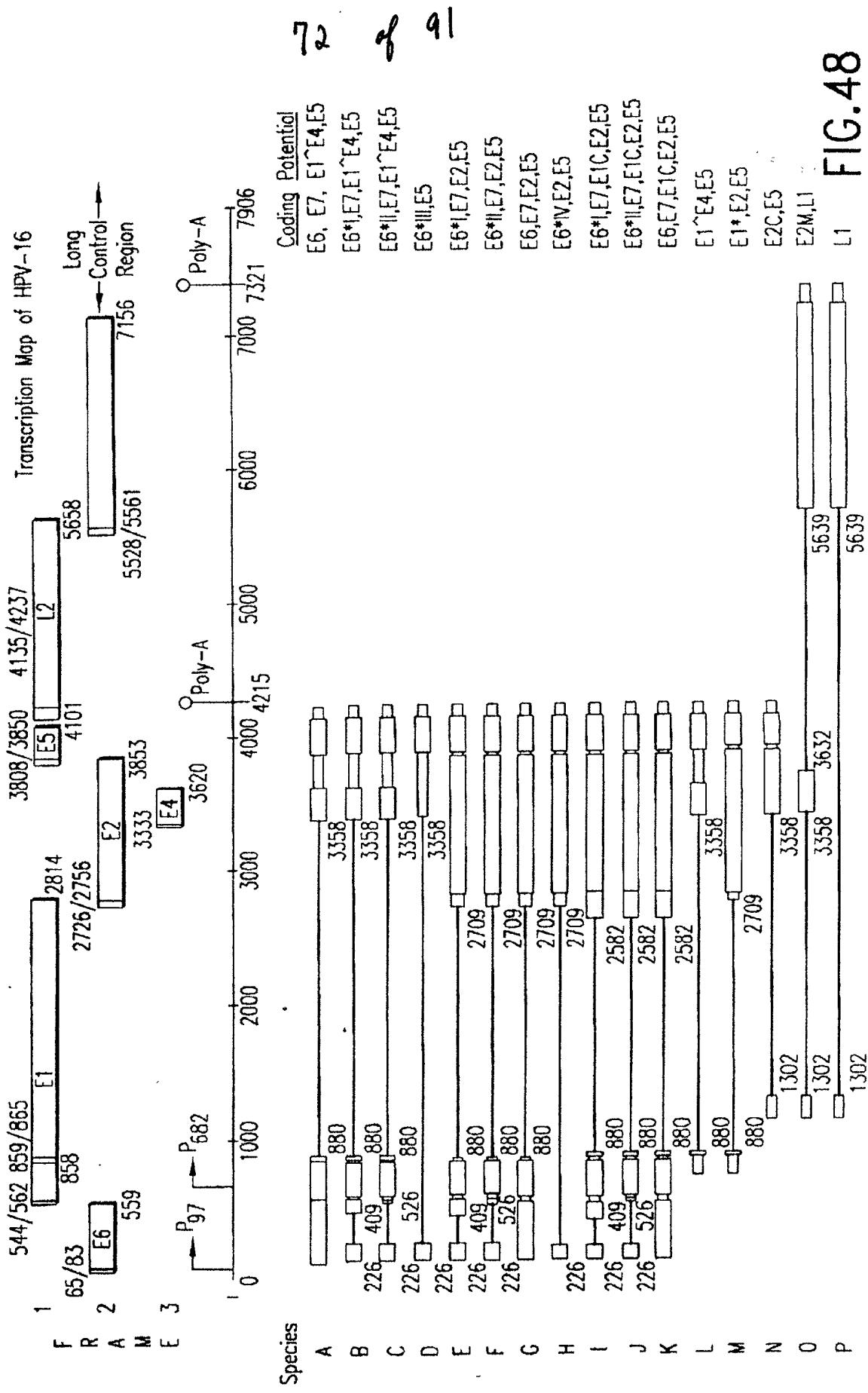


FIG. 47A



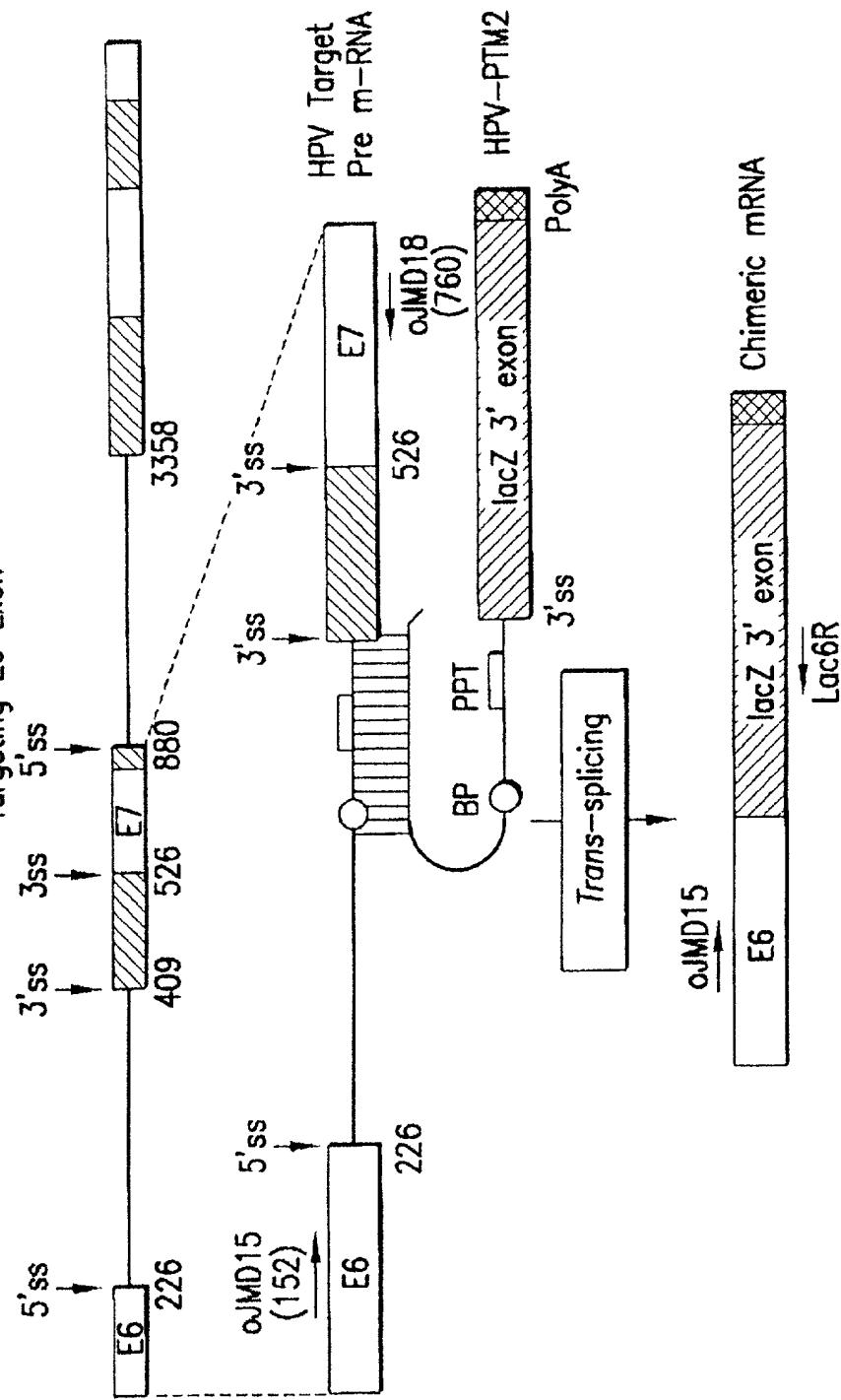
FLAG=C-terminal tag to be used to detect repaired factor VIII protein.

FIG. 47B



73 2 91

SMaRT Strategy to Disrupt the Expression of Human Papillomavirus Type 16



SMaRT Strategy by 3' Exon Replacement: Schematic diagram of HPV-PTM2 binding to the 3' splice site of the HPV type 16 target pre-mRNA

FIG. 49

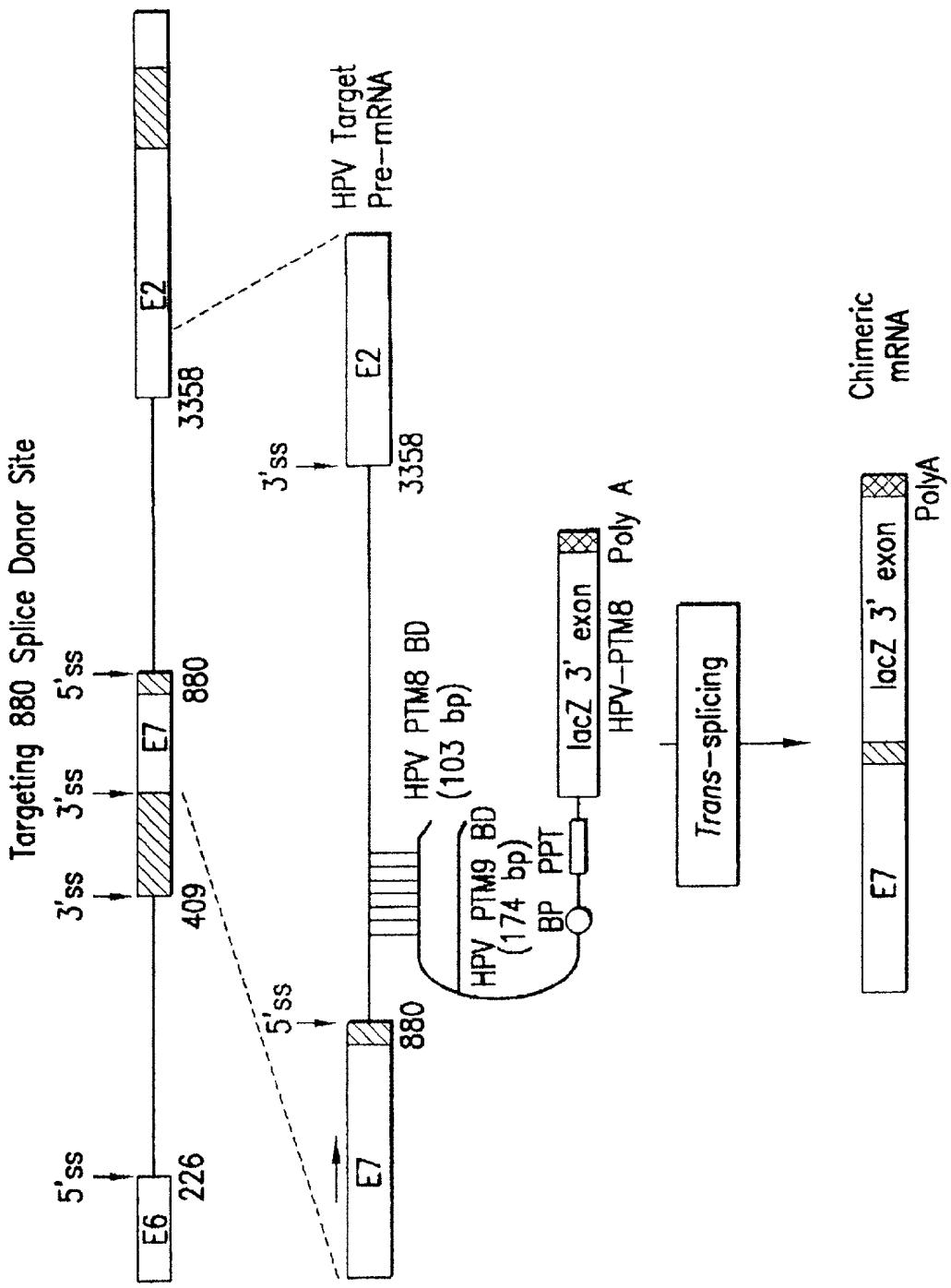


FIG. 50

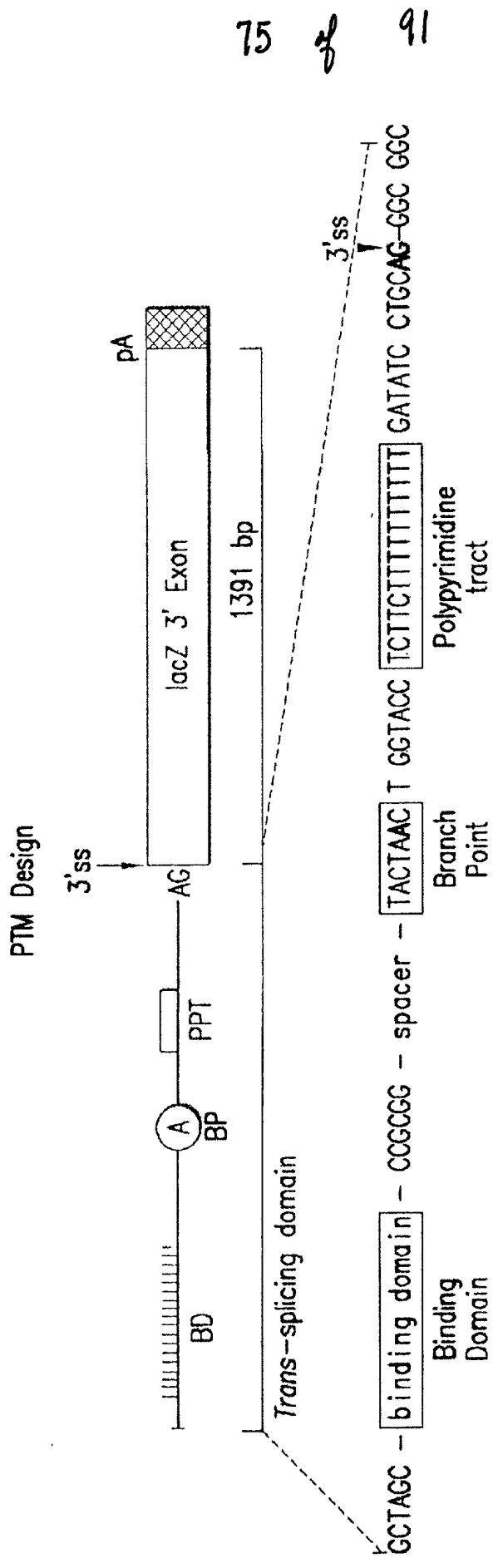


FIG. 51

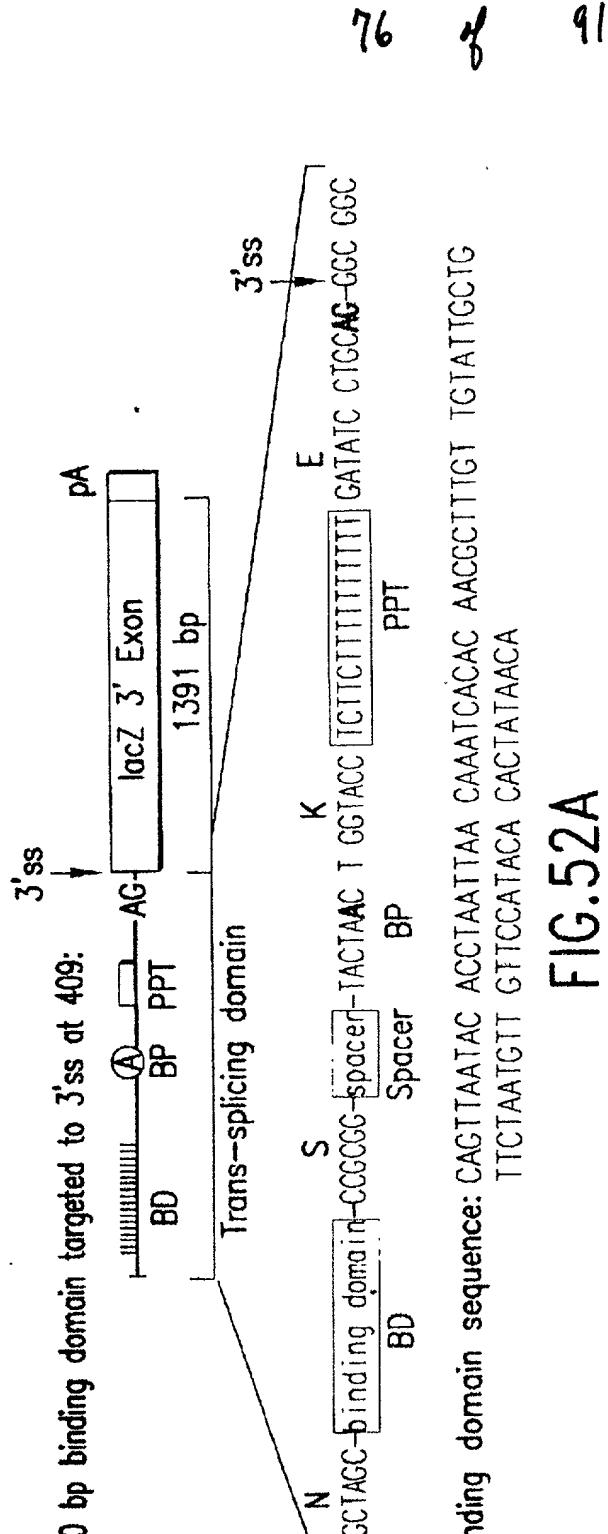
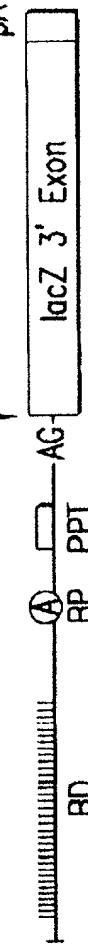


FIG.52A

Binding domain sequence: CAGTTAAC ACTTAATTAA CAAATCACAC AACGCTTTGT TGTATTGCIG
TTCTTAATGTT GTTCCATACA CACTATAACA

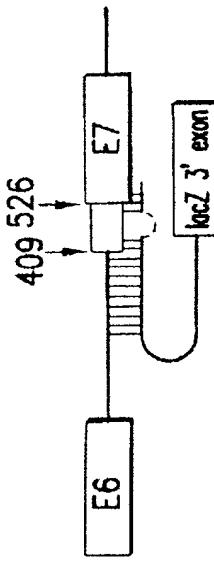
HPV-PTM2 with 80 bp binding domain targeted to 3'ss at 409:
3'ss ↓ pA



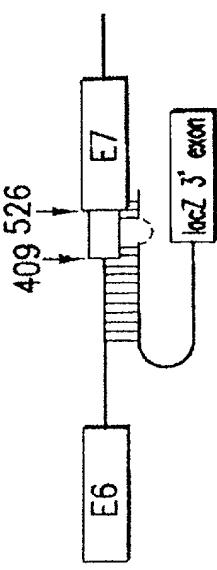
Binding domain sequence: CAGTTAAC ACCTAACCA CAAATCACAC AACGCTTTGT TGTATTGCIG
TTCIAIGTT GTTCCATACA CACTATAACA ATAATGCTA TACTCACTAA
TTTAGAATA AAACTTAA CATTATCAC ATACAGCAT A TCGATTCCC

FIG.52B

FIG. 53



HPV-PTM4 Binding domain (covers both 3'ss at 409 and 526; has 76 bp bubble)
GATGATCTGCAACAAAGACATACATCGACCGGTCCA(53 nt bubble)CTTCAGACACAGTGGCTTTTGAC
AGTTAACACCTAATTAAACAAATCACACAACTGAGTTGTTGTAATGTTGCAGTTCTAACACTAACAT
TAACAAAT



Binding Domains of HPV-PTM3 and 4

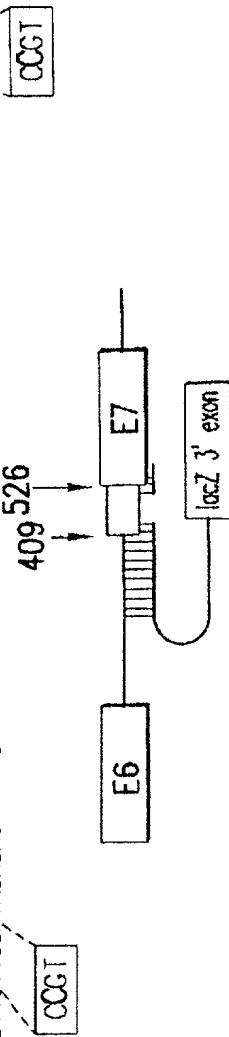
HPV-PTM3 Binding domain (covers both 3'ss at 409 and 526; has 53 bp bubble)
GATGATCTGCAACAAAGACATACATCGACCGGTCCA(53 nt bubble)CTTCAGACACAGTGGCTTTTGAC
AGTTAACACCTAATTAAACAAATCACACAACTGAGTTGTTGTAATGTTGCAGTTCTAACACTAACAT
TAACAAAT

77 526 91

HPV-PTM5 and 6

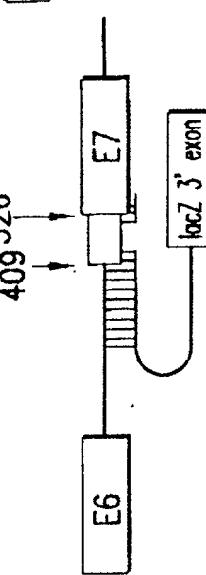
HPV-PTM5. Binding domain (140 nt, has 53 nt bubble, covers 3' ss at position 409 and 526)

GATGATCTGCAAAGACATAACATCGACGGTCCA. CTTCAGGACACAGGGCTTTGACAGTTAACCTAATTAAACAAATCACACAACGGT
TTGTCTATTGGCAGTTCTAACTGTTCCATACACACTAAACA



HPV-PTM6. Binding domain [117-118] has 76 nt bubble covers 3' ss of position 409 and 526)

GATGATCTGAAACAAGAC. GACACAGTGGCTTTGACACTTAATACACCTAATTAAACAAAATCACACAAACGGTTTGTTGTTATGGAGCTTC
AAAGTTGTTGACACACTAAACA
Eos



Note: Nucleotides in bold are modified to prevent PTMs cryptic splicing

FIG. 54

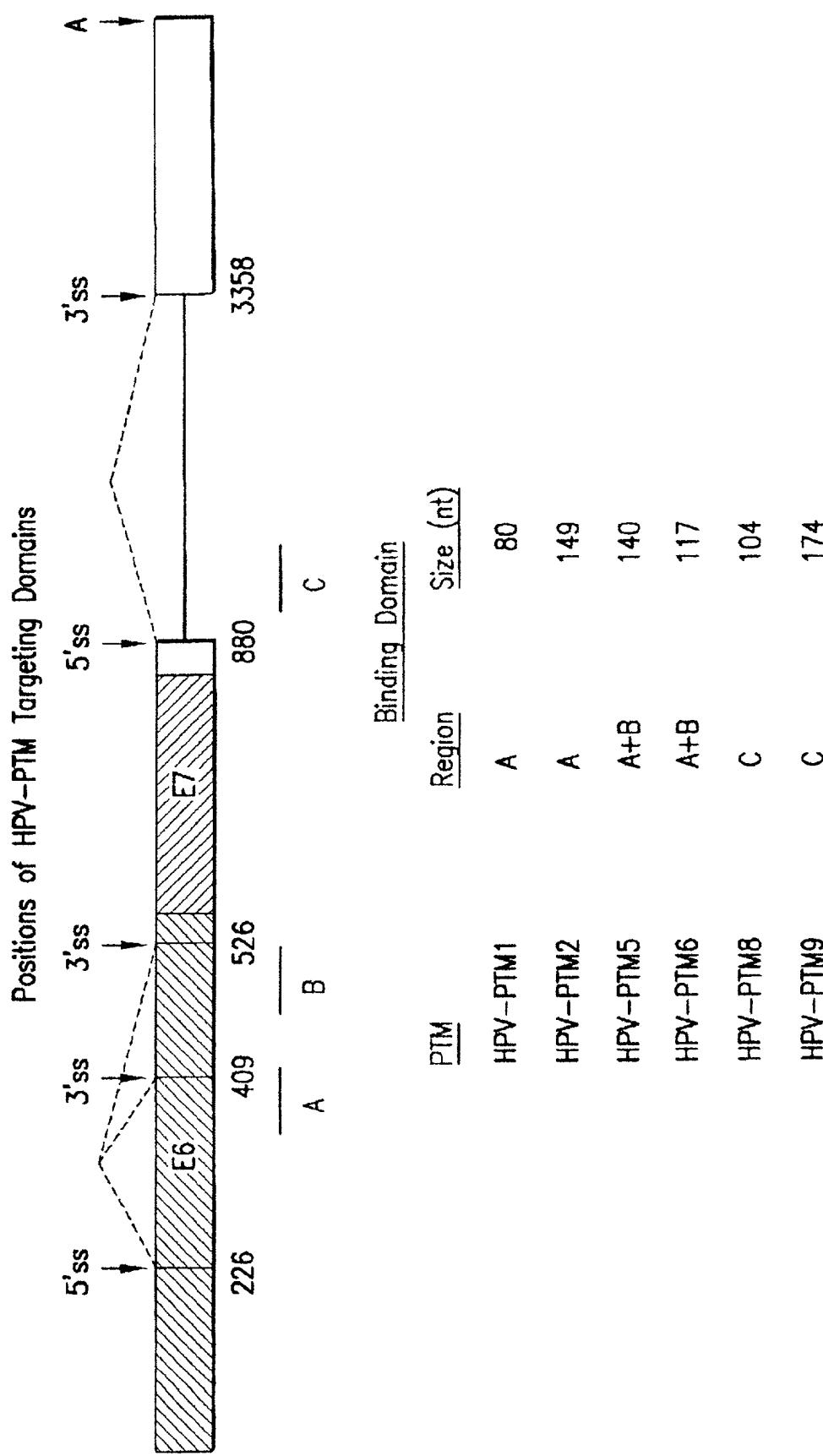
**FIG. 55**

Fig. 56

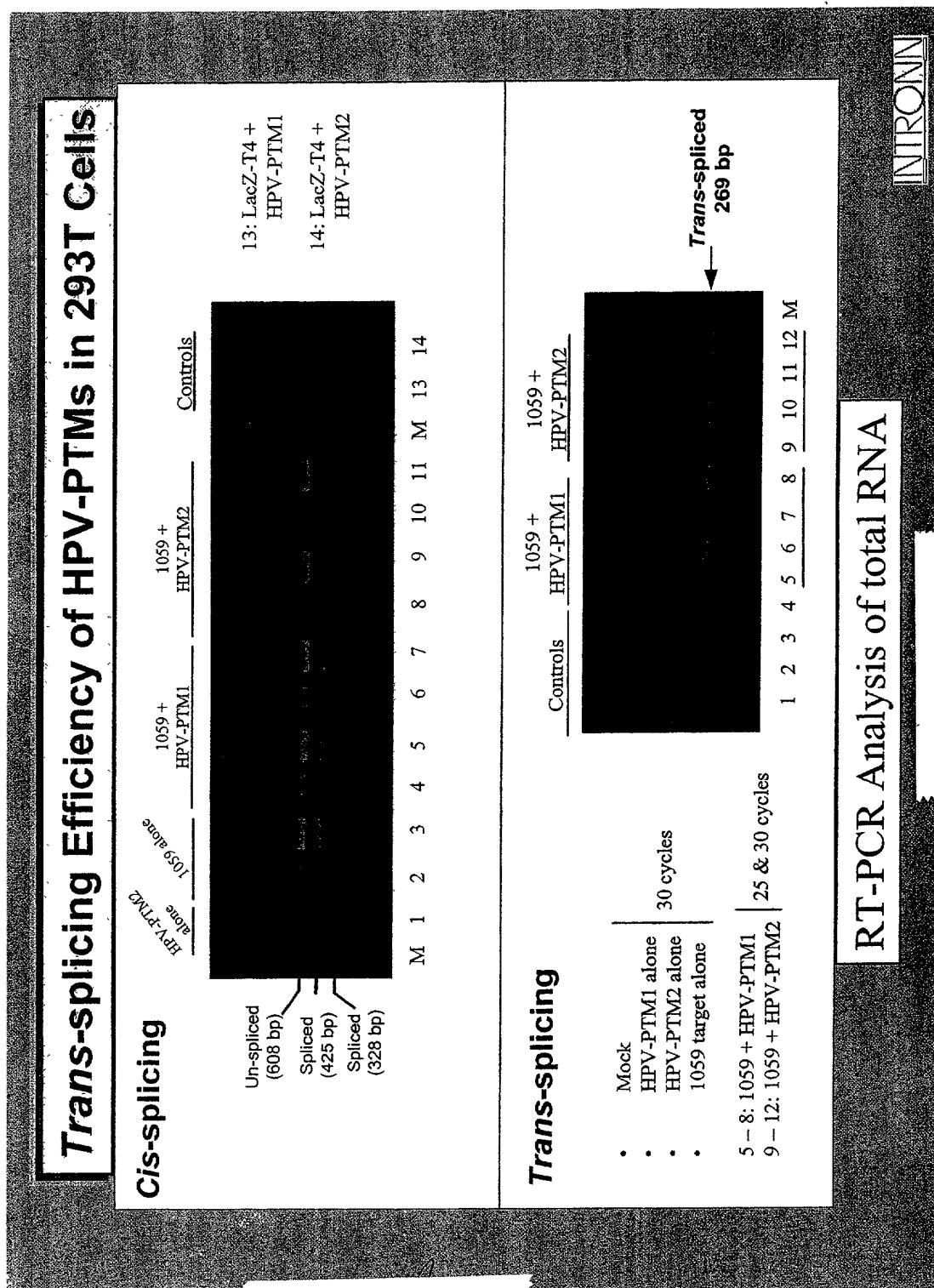
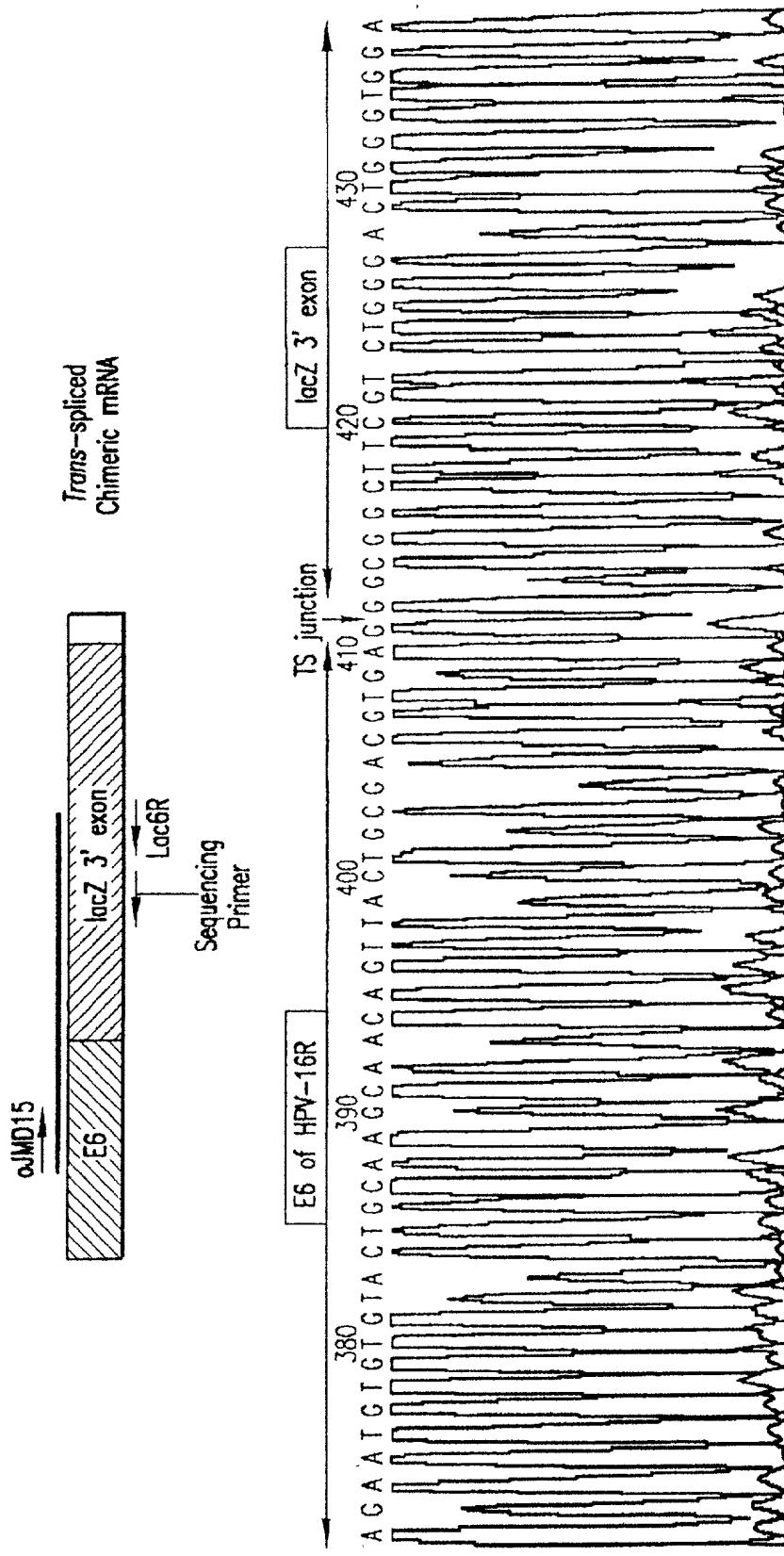


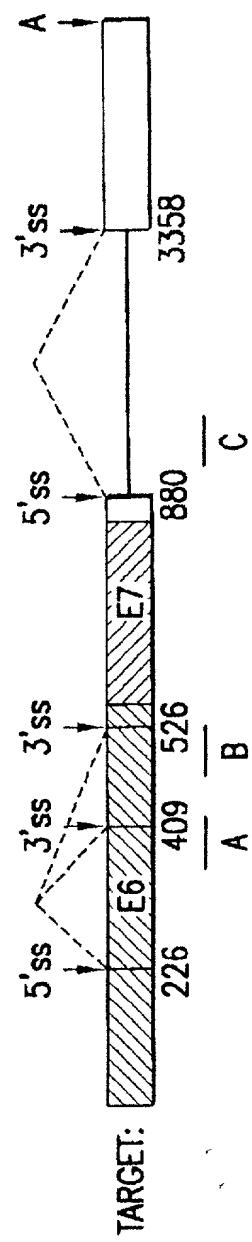
FIG. 57

Trans-splicing between target pre-mRNA and
PTM is accurate (293T cells)



THE BOSTONIAN SOCIETY

Trans-splicing in 293 Cells (Co-transfections)



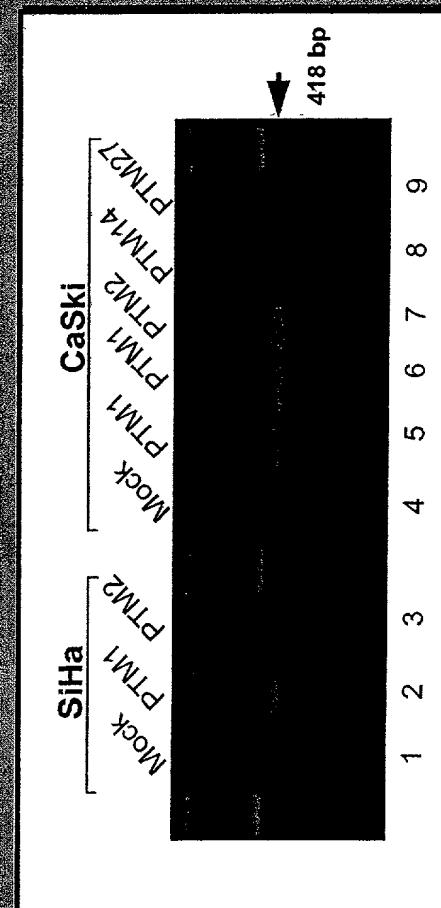
<u>PTM</u>	<u>Binding Domain</u>	<u>% trans-spliced</u>		
	<u>Region</u>	<u>Size (nt)</u>	<u>226 sd</u>	<u>880 sd</u>
HPV-PTM1	A	80	69	0.6
HPV-PTM2	A	149	45	0.9
HPV-PTM5	A+B	140	55	0.8
HPV-PTM5ΔBP/PPT	A+B	140	0.5	0.2
HPV-PTM6	A+B	117	59	1
HPV-PTM8	C	104	7	37
HPV-PTM9	C	174	14	22
CF-PTM27	CF intron	411	0	0

Quantification of Trans-splicing efficiency using real-time QRT-PCR

EIG 58

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Trans-splicing into Endogenous HPV Pre-mRNA Target in SiHa & CaSkI Cells



RT-PCR Conditions

- Total RNA: 400 ng/rxn
- Primer's: oJMD15 + Lac16R
- # Cycles : 35
- Expected product : 418 bp

Details

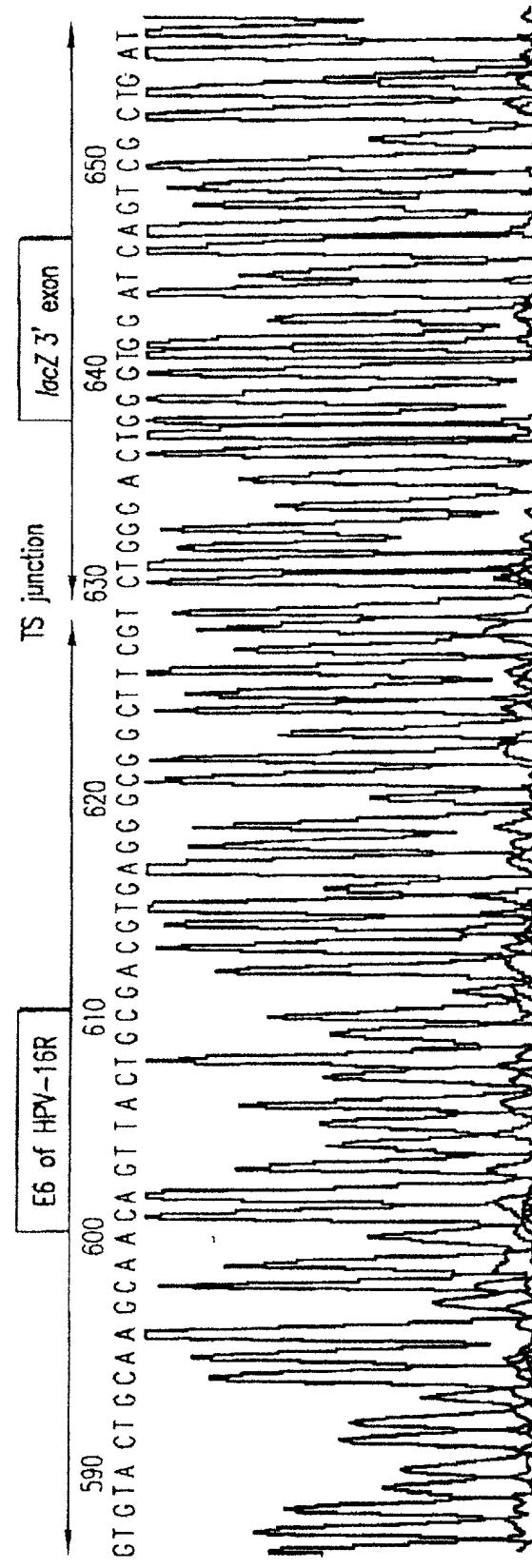
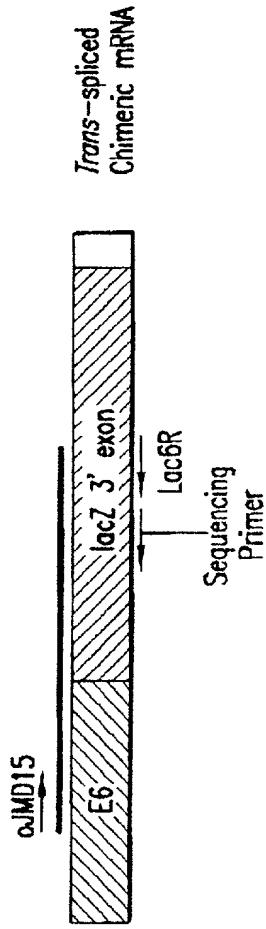
- PTM1, PTM2 : HPV targeted, specific
- PTM14 : CF targeted, non-specific, has 23 bp BD
- PTM14 : CF targeted, non-specific, has 411 bp BD

RT-PCR Analysis of total RNA

INNOM

FIG. 59

*Accurate Trans-splicing of HPV-PTM1
in Si Ha Cells (Endogenous target pre-mRNA)*



Quantification of trans-splicing efficiency using real-time QRT-PCR

FIG. 60

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*Trans-splicing in SiHa Transfections
(Endogenous target)*

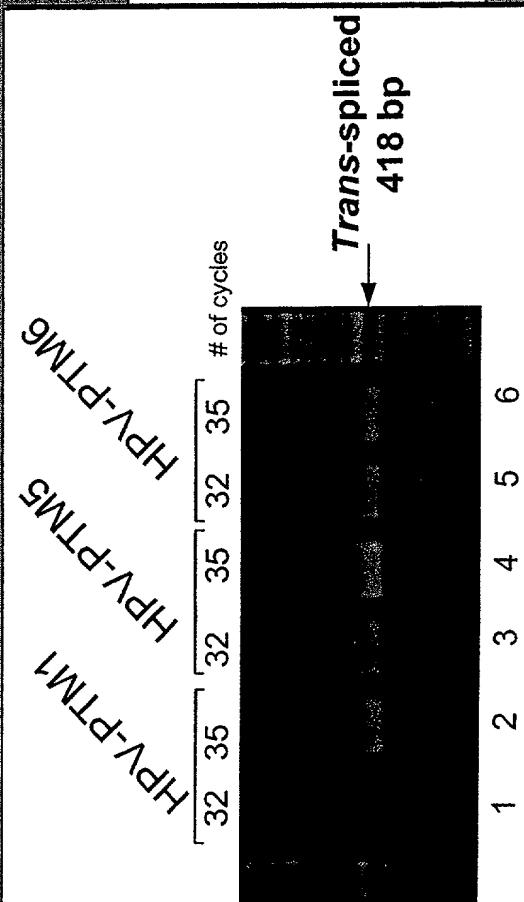
<u>PTM</u>	<u>% trans-spliced</u>
pcDNA3.1	0
HPV-PTM1	0.16
HPV-PTM5	0.12
HPV-PTM6	0.11
CF-PTM27	0

Quantification of *trans-splicing* efficiency using real-time QRT-PCR

FIG.61

86 2 9

Trans-splicing Efficiency of HPV-PTM1, 5, & 6 in SiHa Cells

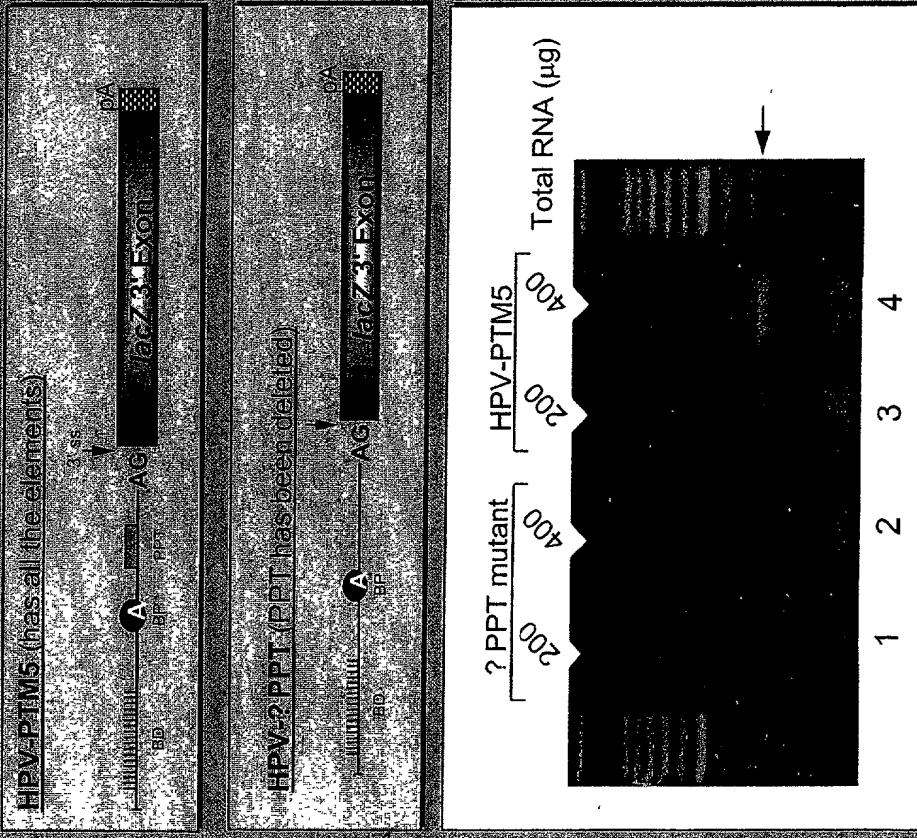


- SiHa cells transfected with 1.5 µg plasmid DNA, Lipofect Plus
- RNA isolated after 48 hr
- Total RNA: 500 ng/Rxn
- Primers: oJMD15 + Lac16R
- Expected product: 418 bp

FIG. 62

NITRONN

Deletion of polypyrimidine tract abolishes trans-splicing



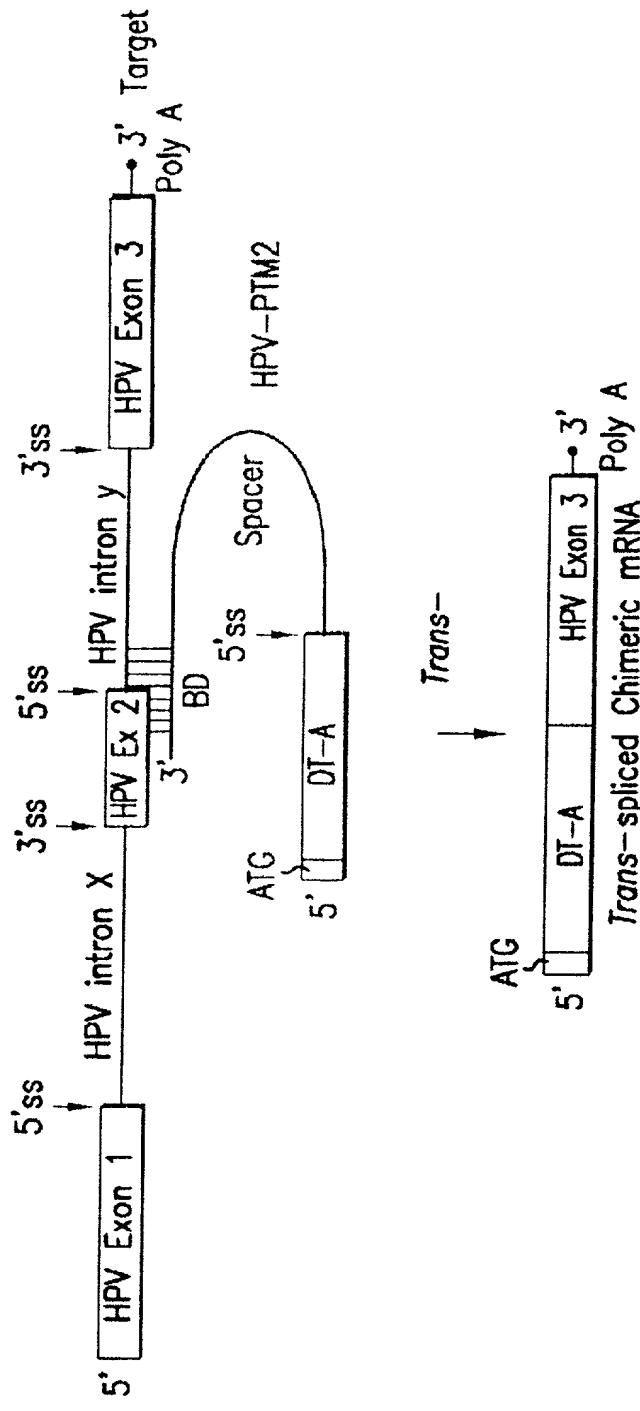
Methods:

- SiHa cells transfected with 1.5 μ g of plasmid DNA
- Total RNA isolated after 48 hr and analyzed by RT-PCR (30 cycles)
 - Primers: oJMD15+Lac6R
 - Expected product: 269 bp
- Lanes 1 & 2: RNA from cells transfected with HPV-? PPT (mutant); No trans-splicing detected
- Lanes 3 & 4: RNA from cells transfected with HPV-PTM5 plasmid; trans-splicing Detected (269 bp product)

INTRON

FIG. 63

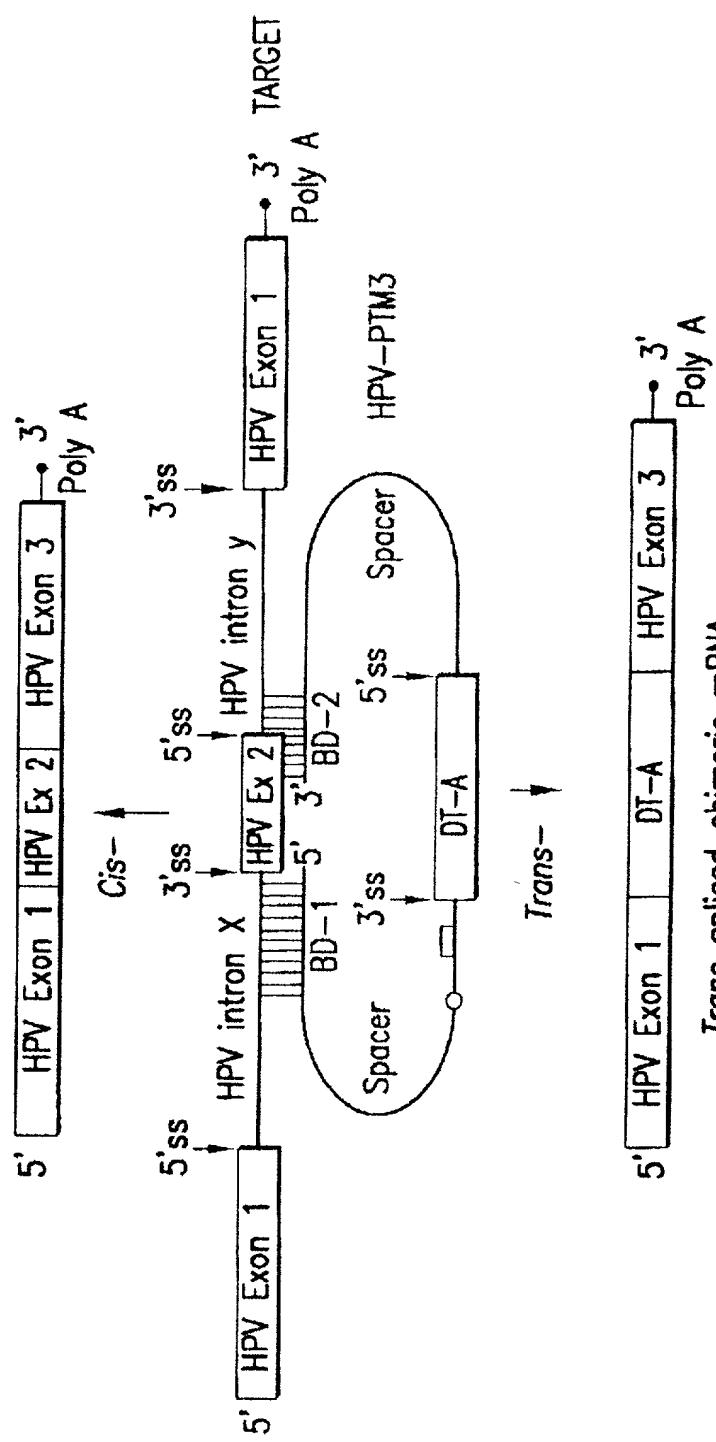
SMART Strategy by 5' Exon Replacement



Schematic diagram of a PTM binding to the 5' splice site of the HPV mini-gene target

FIG.64

Double Trans-splicing



Schematic diagram of a double *Trans-splicing* PTM binding to the 3' and 5' splice sites of the HPV mini-gene target

FIG. 65

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SMaRT Strategy by 3' Exon Replacement: Schematic diagram of a PTM binding to the 3' splice site of the HPV mini-gene target

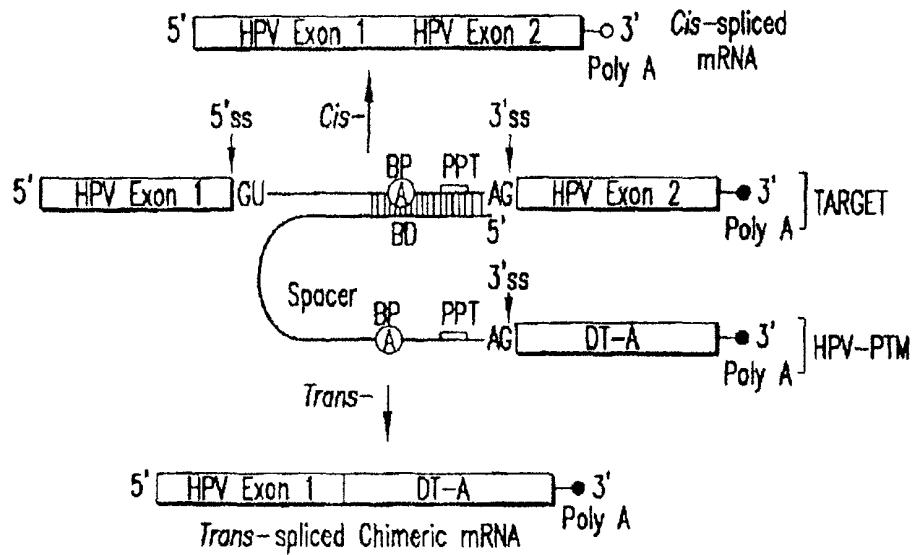


FIG.66A

SMaRT Strategy by 5' Exon Replacement: Schematic diagram of a PTM binding to the 5' splice site of the HPV mini-gene target

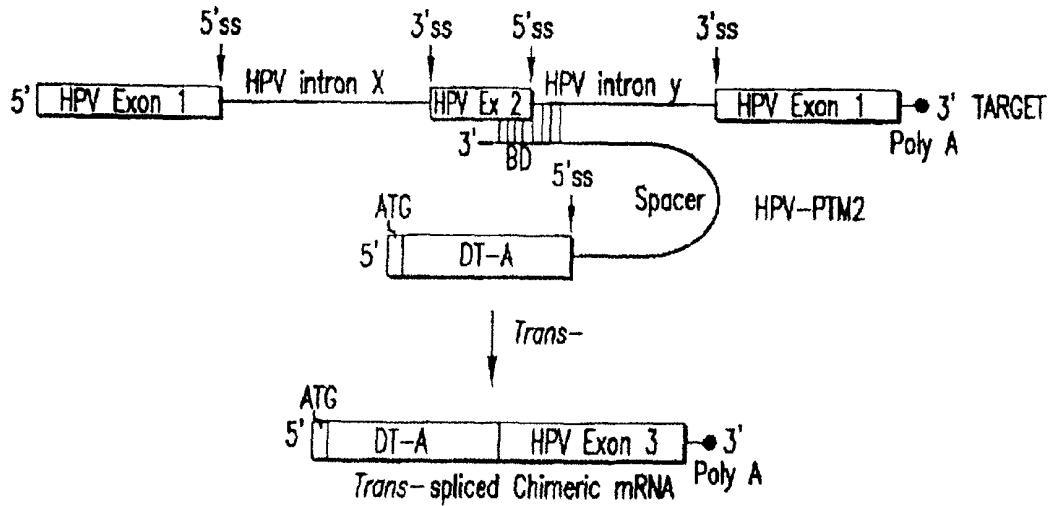


FIG.66B

HPV-PTM3 (For Internal exon replacement)

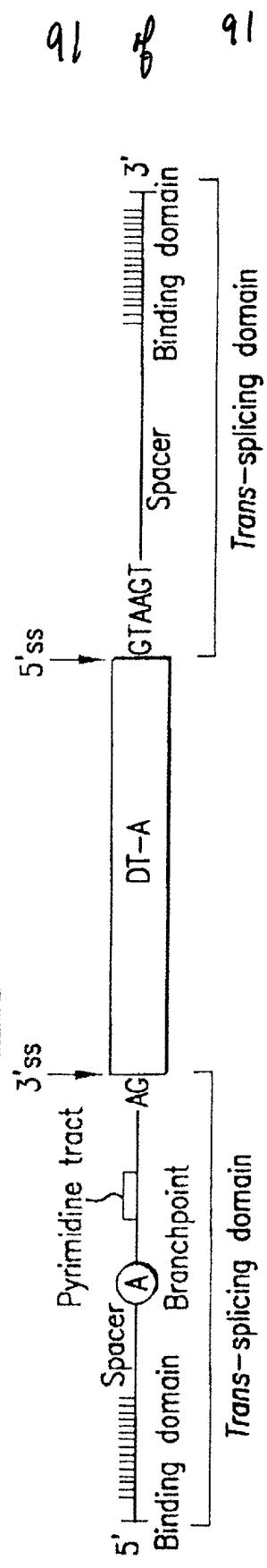


FIG.67